

## Modified Peptides as Therapeutic Agents

### Background of the Invention

Recombinant proteins are an emerging class of therapeutic agents.

- 5 Such recombinant therapeutics have engendered advances in protein formulation and chemical modification. Such modifications can protect therapeutic proteins, primarily by blocking their exposure to proteolytic enzymes. Protein modifications may also increase the therapeutic protein's stability, circulation time, and biological activity. A review
- 10 article describing protein modification and fusion proteins is Francis (1992), Focus on Growth Factors 3:4-10 (Mediscript, London), which is hereby incorporated by reference.

- One useful modification is combination with the "Fc" domain of an antibody. Antibodies comprise two functionally independent parts, a
- 15 variable domain known as "Fab", which binds antigen, and a constant domain known as "Fc", which links to such effector functions as complement activation and attack by phagocytic cells. An Fc has a long serum half-life, whereas an Fab is short-lived. Capon et al. (1989), Nature 337: 525-31. When constructed together with a therapeutic protein, an Fc
- 20 domain can provide longer half-life or incorporate such functions as Fc receptor binding, protein A binding, complement fixation and perhaps even placental transfer. Id. Table 1 summarizes use of Fc fusions known in the art.

**Table 1—Fc fusion with therapeutic proteins**

<b>Form of Fc</b>	<b>Fusion partner</b>	<b>Therapeutic implications</b>	<b>Reference</b>
IgG1	N-terminus of CD30-L	Hodgkin's disease; anaplastic lymphoma; T-cell leukemia	U.S. Patent No. 5,480,981
Murine Fcγ2a	IL-10	anti-inflammatory; transplant rejection	Zheng <i>et al.</i> (1995), <i>J. Immunol.</i> 154: 5590-600
IgG1	TNF receptor	septic shock	Fisher <i>et al.</i> (1996), <i>N. Engl. J. Med.</i> 334: 1697-1702; Van Zee, K. <i>et al.</i> (1996), <i>J. Immunol.</i> 156: 2221-30
IgG, IgA, IgM, or IgE (excluding the first domain)	TNF receptor	inflammation, autoimmune disorders	U.S. Pat. No. 5,808,029, issued September 15, 1998
IgG1	CD4 receptor	AIDS	Capon <i>et al.</i> (1989), <i>Nature</i> 337: 525-31
IgG1, IgG3	N-terminus of IL-2	anti-cancer, antiviral	Harvill <i>et al.</i> (1995), <i>Immunotech.</i> 1: 95-105
IgG1	C-terminus of OPG	osteoarthritis; bone density	WO 97/23614, published July 3, 1997
IgG1	N-terminus of leptin	anti-obesity	PCT/US 97/23183, filed December 11, 1997
Human Ig Cγ1	CTLA-4	autoimmune disorders	Linsley (1991), <i>J. Exp. Med.</i> 174:561-9

- A much different approach to development of therapeutic agents is peptide library screening. The interaction of a protein ligand with its
- 5 receptor often takes place at a relatively large interface. However, as demonstrated for human growth hormone and its receptor, only a few key residues at the interface contribute to most of the binding energy. Clackson *et al.* (1995), *Science* 267: 383-6. The bulk of the protein ligand merely displays the binding epitopes in the right topology or serves
- 10 functions unrelated to binding. Thus, molecules of only "peptide" length (2 to 40 amino acids) can bind to the receptor protein of a given large protein ligand. Such peptides may mimic the bioactivity of the large protein ligand ("peptide agonists") or, through competitive binding, inhibit the bioactivity of the large protein ligand ("peptide antagonists").

Phage display peptide libraries have emerged as a powerful method in identifying such peptide agonists and antagonists. See, for example, Scott *et al.* (1990), *Science* 249: 386; Devlin *et al.* (1990), *Science* 249: 404; U.S. Pat. No. 5,223,409, issued June 29, 1993; U.S. Pat. No. 5,733,731, issued March 31, 1998; U.S. Pat. No. 5,498,530, issued March 12, 1996; U.S. Pat. No. 5,432,018, issued July 11, 1995; U.S. Pat. No. 5,338,665, issued August 16, 1994; U.S. Pat. No. 5,922,545, issued July 13, 1999; WO 96/40987, published December 19, 1996; and WO 98/15833, published April 16, 1998 (each of which is incorporated by reference). In such libraries, random peptide sequences are displayed by fusion with coat proteins of filamentous phage. Typically, the displayed peptides are affinity-eluted against an antibody-immobilized extracellular domain of a receptor. The retained phages may be enriched by successive rounds of affinity purification and repropagation. The best binding peptides may be sequenced to identify key residues within one or more structurally related families of peptides. See, e.g., Cwirla *et al.* (1997), *Science* 276: 1696-9, in which two distinct families were identified. The peptide sequences may also suggest which residues may be safely replaced by alanine scanning or by mutagenesis at the DNA level. Mutagenesis libraries may be created and screened to further optimize the sequence of the best binders. Lowman (1997), *Ann. Rev. Biophys. Biomol. Struct.* 26: 401-24.

Structural analysis of protein-protein interaction may also be used to suggest peptides that mimic the binding activity of large protein ligands. In such an analysis, the crystal structure may suggest the identity and relative orientation of critical residues of the large protein ligand, from which a peptide may be designed. See, e.g., Takasaki *et al.* (1997), *Nature Biotech.* 15: 1266-70. These analytical methods may also be used to investigate the interaction between a receptor protein and peptides

selected by phage display, which may suggest further modification of the peptides to increase binding affinity.

- Other methods compete with phage display in peptide research. A peptide library can be fused to the carboxyl terminus of the lac repressor and expressed in E. coli. Another E. coli-based method allows display on the cell's outer membrane by fusion with a peptidoglycan-associated lipoprotein (PAL). Hereinafter, these and related methods are collectively referred to as "E. coli display." In another method, translation of random RNA is halted prior to ribosome release, resulting in a library of polypeptides with their associated RNA still attached. Hereinafter, this and related methods are collectively referred to as "ribosome display." Other methods employ chemical linkage of peptides to RNA; see, for example, Roberts & Szostak (1997), Proc. Natl. Acad. Sci. USA, 94: 12297-303. Hereinafter, this and related methods are collectively referred to as "RNA-peptide screening." Chemically derived peptide libraries have been developed in which peptides are immobilized on stable, non-biological materials, such as polyethylene rods or solvent-permeable resins. Another chemically derived peptide library uses photolithography to scan peptides immobilized on glass slides. Hereinafter, these and related methods are collectively referred to as "chemical-peptide screening." Chemical-peptide screening may be advantageous in that it allows use of D-amino acids and other unnatural analogues, as well as non-peptide elements. Both biological and chemical methods are reviewed in Wells & Lowman (1992), Curr. Opin. Biotechnol. 3: 355-62.
- Conceptually, one may discover peptide mimetics of any protein using phage display and the other methods mentioned above. These methods have been used for epitope mapping, for identification of critical amino acids in protein-protein interactions, and as leads for the discovery of new therapeutic agents. E.g., Cortese et al. (1996), Curr. Opin. Biotech. 7:

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616-21. Peptide libraries are now being used most often in immunological studies, such as epitope mapping. Kreeger (1996), The Scientist 10(13): 19-20.

Of particular interest here is use of peptide libraries and other techniques in the discovery of pharmacologically active peptides. A number of such peptides identified in the art are summarized in Table 2. The peptides are described in the listed publications, each of which is hereby incorporated by reference. The pharmacologic activity of the peptides is described, and in many instances is followed by a shorthand term therefor in parentheses. Some of these peptides have been modified (e.g., to form C-terminally cross-linked dimers). Typically, peptide libraries were screened for binding to a receptor for a pharmacologically active protein (e.g., EPO receptor). In at least one instance (CTLA4), the peptide library was screened for binding to a monoclonal antibody.

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Table 2—Pharmacologically active peptides

Form of peptide	Binding partner/ protein of interest <sup>a</sup>	Pharmacologic activity	Reference
intrapeptide disulfide-bonded	EPO receptor	EPO-mimetic	Wrighton <i>et al.</i> (1996), <i>Science</i> 273: 458-63; U.S. Pat. No. 5,773,569, issued June 30, 1998 to Wrighton <i>et al.</i>
C-terminally cross-linked dimer	EPO receptor	EPO-mimetic	Livnah <i>et al.</i> (1996), <i>Science</i> 273: 464-71; Wrighton <i>et al.</i> (1997), <i>Nature Biotechnology</i> 15: 1261-5; International patent application WO 96/40772, published Dec. 19, 1996
linear	EPO receptor	EPO-mimetic	Naranda <i>et al.</i> (1999), <i>Proc. Natl. Acad. Sci. USA</i> , 96: 7569-74
linear	c-Mpl	TPO-mimetic	Cwiria <i>et al.</i> (1997) <i>Science</i> 276: 1696-9; U.S. Pat. No. 5,869,451, issued Feb. 9, 1999; U.S. Pat. No. 5,932,946, issued Aug. 3, 1999
C-terminally cross-linked dimer	c-Mpl	TPO-mimetic	Cwiria <i>et al.</i> (1997), <i>Science</i> 276: 1696-9
disulfide-linked dimer		stimulation of hematopoiesis ("G-CSF-mimetic")	Paukovits <i>et al.</i> (1984), <i>Hoppe-Seviers Z. Physiol. Chem.</i> 365: 303-11; Laerum <i>et al.</i> (1988), <i>Exp. Hemat.</i> 16: 274-80
alkylene-linked dimer		G-CSF-mimetic	Bhatnagar <i>et al.</i> (1996), <i>J. Med. Chem.</i> 39: 3814-9; Cuthbertson <i>et al.</i> (1997), <i>J. Med. Chem.</i> 40: 2876-82; King <i>et al.</i> (1991), <i>Exp. Hematol.</i> 19:481; King <i>et al.</i> (1995), <i>Blood</i> 86 (Suppl. 1): 309a
linear	IL-1 receptor	inflammatory and autoimmune diseases ("IL-1 antagonist" or "IL-1ra-mimetic")	U.S. Pat. No. 5,608,035; U.S. Pat. No. 5,786,331; U.S. Pat. No. 5,880,096; Yanofsky <i>et al.</i> (1996), <i>Proc. Natl. Acad. Sci.</i> 93: 7381-6; Akeson <i>et al.</i> (1996), <i>J. Biol. Chem.</i>

<sup>a</sup> The protein listed in this column may be bound by the associated peptide (e.g., EPO receptor, IL-1 receptor) or mimicked by the associated peptide. The references listed for each clarify whether the molecule is bound by or mimicked by the peptides.

linear	Facteur thymique serique (FTS)	stimulation of lymphocytes ("FTS-mimetic")	271: 30517-23; Wiekzorek <i>et al.</i> (1997), <i>Pol. J. Pharmacol.</i> 49: 107-17; Yanofsky (1996), <i>PNAs</i> , 93:7381-7386.
intrapeptide disulfide bonded	CTLA4 MAb	CTLA4-mimetic	Inagaki-Ohara <i>et al.</i> (1996), <i>Cellular Immunol.</i> 171: 30-40; Yoshida (1984), <i>Int. J. Immunopharmacol.</i> 6:141-6.
exocyclic	TNF- $\alpha$ receptor	TNF- $\alpha$ antagonist	Fukumoto <i>et al.</i> (1998), <i>Nature Biotech.</i> 16: 267-70
linear	TNF- $\alpha$ receptor	TNF- $\alpha$ antagonist	Takasaki <i>et al.</i> (1997), <i>Nature Biotech.</i> 15:1266-70; WO 98/53842, published December 3, 1998
intrapeptide disulfide bonded	C3b	inhibition of complement activation; autoimmune diseases ("C3b-antagonist")	Chirinos-Rojas ( ), <i>J. Imm.</i> , 5621-5626.
linear	vinculin	cell adhesion processes—cell growth, differentiation, wound healing, tumor metastasis ("vinculin binding")	Sahu <i>et al.</i> (1996), <i>J. Immunol.</i> 157: 884-91; Morikis <i>et al.</i> (1998), <i>Protein Sci.</i> 7: 619-27
linear	C4 binding protein (C4BP)	anti-thrombotic	Adey <i>et al.</i> (1997), <i>Biochem. J.</i> 324: 523-8
linear	urokinase receptor	processes associated with urokinase interaction with its receptor (e.g., angiogenesis, tumor cell invasion and metastasis); ("UKR antagonist")	Linse <i>et al.</i> (1997), <i>J. Biol. Chem.</i> 272: 14658-65
linear	Mdm2, Hdm2	Inhibition of inactivation of p53 mediated by Mdm2 or hdm2; anti-tumor ("Mdm/hdm antagonist")	Goodson <i>et al.</i> (1994), <i>Proc. Natl. Acad. Sci.</i> 91: 7129-33; International application WO 97/35969, published October 2, 1997
linear	p21 <sup>WAF1</sup>	anti-tumor by mimicking the activity of p21 <sup>WAF1</sup>	Picksley <i>et al.</i> (1994), <i>Oncogene</i> 9: 2523-9; Bottger <i>et al.</i> (1997) <i>J. Mol. Biol.</i> 269: 744-56; Bottger <i>et al.</i> (1996), <i>Oncogene</i> 13: 2141-7
linear	farnesyl transferase	anti-cancer by preventing activation of ras oncogene	Ball <i>et al.</i> (1997), <i>Curr. Biol.</i> 7: 71-80
linear	Ras effector domain	anti-cancer by inhibiting biological function of the ras oncogene	Gibbs <i>et al.</i> (1994), <i>Cell</i> 77:175-178
linear	SH2/SH3	anti-cancer by inhibiting	Moodie <i>et al.</i> (1994), <i>Trends Genet</i> 10: 44-48
			Rodriguez <i>et al.</i> (1994), <i>Nature</i> 370:527-532
			Pawson <i>et al.</i> (1993),

<sup>a</sup> FTS is a thymic hormone mimicked by the molecule of this invention rather than a receptor bound by the molecule of this invention.

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	domains	tumor growth with activated tyrosine kinases	<u>Curr. Biol.</u> 3:434-432 Yu et al. (1994), <u>Cell</u> 76:933-945
linear	p16 <sup>INK4</sup>	anti-cancer by mimicking activity of p16; e.g., inhibiting cyclin D-Cdk complex ("p16-mimetic")	Fähræus et al. (1996), <u>Curr. Biol.</u> 6:84-91
linear	Src, Lyn	inhibition of Mast cell activation, IgE-related conditions, type I hypersensitivity ("Mast cell antagonist")	Stauffer et al. (1997), <u>Biochem.</u> 36: 9388-94
linear	Mast cell protease	treatment of inflammatory disorders mediated by release of tryptase-6 ("Mast cell protease inhibitors")	International application WO 98/33812, published August 6, 1998
linear	SH3 domains	treatment of SH3-mediated disease states ("SH3 antagonist")	Rickles et al. (1994), <u>EMBO J.</u> 13: 5598-5604; Sparks et al. (1994), <u>J. Biol. Chem.</u> 269: 23853-6; Sparks et al. (1996), <u>Proc. Natl. Acad. Sci.</u> 93: 1540-4
linear	HBV core antigen (HBcAg)	treatment of HBV viral infections ("anti-HBV")	Dyson & Muray (1995), <u>Proc. Natl. Acad. Sci.</u> 92: 2194-8
linear	selectins	neutrophil adhesion; inflammatory diseases ("selectin antagonist")	Martens et al. (1995), <u>J. Biol. Chem.</u> 270: 21129-36; European patent application EP 0 714 912, published June 5, 1996
linear, cyclized	calmodulin	calmodulin antagonist	Pierce et al. (1995), <u>Molec. Diversity</u> 1: 259-65; Dedman et al. (1993), <u>J. Biol. Chem.</u> 268: 23025-30; Adey & Kay (1996), <u>Gene</u> 169: 133-4
linear, cyclized-	integrins	tumor-homing; treatment for conditions related to integrin-mediated cellular events, including platelet aggregation, thrombosis, wound healing, osteoporosis, tissue repair, angiogenesis (e.g., for treatment of cancer), and tumor invasion ("integrin-binding")	International applications WO 95/14714, published June 1, 1995; WO 97/08203, published March 6, 1997; WO 98/10795, published March 19, 1998; WO 99/24462, published May 20, 1999; Kraft et al. (1999), <u>J. Biol. Chem.</u> 274: 1979-1985
cyclic, linear	fibronectin and extracellular matrix components of T cells and macrophages	treatment of inflammatory and autoimmune conditions	WO 98/09985, published March 12, 1998



linear	somatostatin and cortistatin	treatment or prevention of hormone-producing tumors, acromegaly, gigantism, dementia, gastric ulcer, tumor growth, inhibition of hormone secretion, modulation of sleep or neural activity	European patent application 0 911 393, published April 28, 1999
linear	bacterial lipopolysaccharide	antibiotic; septic shock; disorders modulatable by CAP37	U.S. Pat. No. 5,877,151, issued March 2, 1999
linear or cyclic, including D-amino acids	pardaxin, mellitin	antipathogenic	WO 97/31019, published 28 August 1997
linear, cyclic	VIP	impotence, neurodegenerative disorders	WO 97/40070, published October 30, 1997
linear	CTLs	cancer	EP 0 770 624, published May 2, 1997
linear	THF-gamma2		Burnstein (1988), <i>Biochem.</i> , 27:4066-71.
linear	Amylin		Cooper (1987), <i>Proc. Natl. Acad. Sci.</i> , 84:8628-32.
linear	Adrenomedullin		Kitamura (1993), <i>BBRC</i> , 192:553-60.
cyclic, linear	VEGF	anti-angiogenic; cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis ("VEGF antagonist")	Fairbrother (1998), <i>Biochem.</i> , 37:17754-17764.
cyclic	MMP	inflammation and autoimmune disorders; tumor growth ("MMP inhibitor")	Koivunen (1999), <i>Nature Biotech.</i> , 17:768-774.
	HGH fragment		U.S. Pat. No. 5,869,452
	Echistatin	inhibition of platelet aggregation	Gan (1988), <i>J. Biol. Chem.</i> , 263:19827-32.
linear	SLE autoantibody	SLE	WO 96/30057, published October 3, 1996
	GD1alpha	suppression of tumor metastasis	Ishikawa et al. (1998), <i>FEBS Lett.</i> 441 (1): 20-4
	antiphospholipid beta-2-glycoprotein-I (β2GPI) antibodies	endothelial cell activation, antiphospholipid syndrome (APS), thromboembolic phenomena, thrombocytopenia, and recurrent fetal loss	Blank et al. (1999), <i>Proc. Natl. Acad. Sci. USA</i> 96: 5154-8
linear	T Cell Receptor beta chain	diabetes	WO 96/11214, published April 18, 1996

- Peptides identified by peptide library screening have been regarded as “leads” in development of therapeutic agents rather than as therapeutic agents themselves. Like other proteins and peptides, they would be rapidly removed in vivo either by renal filtration, cellular clearance mechanisms in the reticuloendothelial system, or proteolytic degradation. Francis (1992), Focus on Growth Factors 3: 4-11. As a result, the art presently uses the identified peptides to validate drug targets or as scaffolds for design of organic compounds that might not have been as easily or as quickly identified through chemical library screening.
- 10 Lowman (1997), Ann. Rev. Biophys. Biomol. Struct. 26: 401-24; Kay et al. (1998), Drug Disc. Today 3: 370-8. The art would benefit from a process by which such peptides could more readily yield therapeutic agents.

#### Summary of the Invention

- The present invention concerns a process by which the in vivo half-life of one or more biologically active peptides is increased by fusion with a vehicle. In this invention, pharmacologically active compounds are prepared by a process comprising:
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- a) selecting at least one peptide that modulates the activity of a protein of interest; and
- 20 b) preparing a pharmacologic agent comprising at least one vehicle covalently linked to at least one amino acid sequence of the selected peptide.

- The preferred vehicle is an Fc domain. The peptides screened in step (a) are preferably expressed in a phage display library. The vehicle and the peptide may be linked through the N- or C-terminus of the peptide or the vehicle, as described further below. Derivatives of the above compounds (described below) are also encompassed by this invention.
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The compounds of this invention may be prepared by standard synthetic methods, recombinant DNA techniques, or any other methods of

preparing peptides and fusion proteins. Compounds of this invention that encompass non-peptide portions may be synthesized by standard organic chemistry reactions, in addition to standard peptide chemistry reactions when applicable.

- 5           The primary use contemplated is as therapeutic or prophylactic agents. The vehicle-linked peptide may have activity comparable to—or even greater than—the natural ligand mimicked by the peptide. In addition, certain natural ligand-based therapeutic agents might induce antibodies against the patient's own endogenous ligand; the vehicle-linked  
10 peptide avoids this pitfall by having little or typically no sequence identity with the natural ligand.

- Although mostly contemplated as therapeutic agents, compounds of this invention may also be useful in screening for such agents. For example, one could use an Fc-peptide (e.g., Fc-SH2 domain peptide) in an  
15 assay employing anti-Fc coated plates. The vehicle, especially Fc, may make insoluble peptides soluble and thus useful in a number of assays.

- The compounds of this invention may be used for therapeutic or prophylactic purposes by formulating them with appropriate pharmaceutical carrier materials and administering an effective amount to  
20 a patient, such as a human (or other mammal) in need thereof. Other related aspects are also included in the instant invention.

Numerous additional aspects and advantages of the present invention will become apparent upon consideration of the figures and detailed description of the invention.

#### 25           **Brief Description of the Figures**

Figure 1 shows a schematic representation of an exemplary process of the invention. In this preferred process, the vehicle is an Fc domain, which is linked to the peptide covalently by expression from a DNA

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construct encoding both the Fc domain and the peptide. As noted in Figure 1, the Fc domains spontaneously form a dimer in this process.

Figure 2 shows exemplary Fc dimers that may be derived from an IgG1 antibody. "Fc" in the figure represents any of the Fc variants within the meaning of "Fc domain" herein. "X<sup>1</sup>" and "X<sup>2</sup>" represent peptides or linker-peptide combinations as defined hereinafter. The specific dimers are as follows:

A, D: Single disulfide-bonded dimers. IgG1 antibodies typically have two disulfide bonds at the hinge region between the constant and variable domains. The Fc domain in Figures 2A and 2 D may be formed by truncation between the two disulfide bond sites or by substitution of a cysteinyl residue with an unreactive residue (e.g., alanyl). In Figure 2A, the Fc domain is linked at the amino terminus of the peptides; in 2D, at the carboxyl terminus.

15 B, E: Doubly disulfide-bonded dimers. This Fc domain may be formed by truncation of the parent antibody to retain both cysteinyl residues in the Fc domain chains or by expression from a construct including a sequence encoding such an Fc domain. In Figure 2B, the Fc domain is linked at the amino terminus of the peptides; in 2E, at the carboxyl terminus.

20 C, F: Noncovalent dimers. This Fc domain may be formed by elimination of the cysteinyl residues by either truncation or substitution. One may desire to eliminate the cysteinyl residues to avoid impurities formed by reaction of the cysteinyl residue with cysteinyl residues of other proteins present in the host cell. The noncovalent bonding of the Fc domains is sufficient to hold together the dimer. Other dimers may be formed by using Fc domains derived from different types of antibodies (e.g., IgG2, IgM).

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Figure 3 shows the structure of preferred compounds of the invention that feature tandem repeats of the pharmacologically active peptide. Figure 3A shows a single chain molecule and may also represent the DNA construct for the molecule. Figure 3B shows a dimer in which the linker-peptide portion is present on only one chain of the dimer. Figure 3C shows a dimer having the peptide portion on both chains. The dimer of Figure 3C will form spontaneously in certain host cells upon expression of a DNA construct encoding the single chain shown in Figure 3A. In other host cells, the cells could be placed in conditions favoring formation of dimers or the dimers can be formed in vitro.

Figure 4 shows exemplary nucleic acid and amino acid sequences (SEQ ID NOS: 1 and 2, respectively) of human IgG1 Fc that may be used in this invention.

Figure 5 shows a synthetic scheme for the preparation of PEGylated peptide 19 (SEQ ID NO: 3).

Figure 6 shows a synthetic scheme for the preparation of PEGylated peptide 20 (SEQ ID NO: 4).

Figure 7 shows the nucleotide and amino acid sequences (SEQ ID NOS: 5 and 6, respectively) of the molecule identified as "Fc-TMP" in Example 2 hereinafter.

Figure 8 shows the nucleotide and amino acid sequences (SEQ. ID. NOS: 7 and 8, respectively) of the molecule identified as "Fc-TMP-TMP" in Example 2 hereinafter.

Figure 9 shows the nucleotide and amino acid sequences (SEQ. ID. NOS: 9 and 10, respectively) of the molecule identified as "TMP-TMP-Fc" in Example 2 hereinafter.

Figure 10 shows the nucleotide and amino acid sequences (SEQ. ID. NOS: 11 and 12, respectively) of the molecule identified as "TMP-Fc" in Example 2 hereinafter.

Figure 11 shows the number of platelets generated in vivo in normal female BDF1 mice treated with one 100 µg/kg bolus injection of various compounds, with the terms defined as follows.

- 5            PEG-MGDF: 20 kD average molecular weight PEG attached by reductive amination to the N-terminal amino group of amino acids 1-163 of native human TPO, which is expressed in E. coli (so that it is not glycosylated);
- TMP: the TPO-mimetic peptide having the amino acid sequence IEGPTLRQWLAARA (SEQ ID NO: 13);
- 10          TMP-TMP: the TPO-mimetic peptide having the amino acid sequence IEGPTLRQWLAARA-GGGGGGGG-IEGPTLRQWLAARA (SEQ ID NO: 14);
- PEG-TMP-TMP: the peptide of SEQ ID NO: 14, wherein the PEG group is a 5 kD average molecular weight PEG attached as
- 15          shown in Figure 6;
- Fc-TMP-TMP: the compound of SEQ ID NO: 8 (Figure 8) dimerized with an identical second monomer (i.e., Cys residues 7 and 10 are bound to the corresponding Cys residues in the second monomer to form a dimer, as shown in Figure 2); and
- 20          TMP-TMP-Fc is the compound of SEQ ID NO: 10 (Figure 9) dimerized in the same way as TMP-TMP-Fc except that the Fc domain is attached at the C-terminal end rather than the N-terminal end of the TMP-TMP peptide.

25          Figure 12 shows the number of platelets generated in vivo in normal BDF1 mice treated with various compounds delivered via implanted osmotic pumps over a 7-day period. The compounds are as defined for Figure 7.

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Figure 13 shows the nucleotide and amino acid sequences (SEQ ID NOS: 15 and 16, respectively) of the molecule identified as "Fc-EMP" in Example 3 hereinafter.

Figure 14 shows the nucleotide and amino acid sequences (SEQ ID NOS: 17 and 18, respectively) of the molecule identified as "EMP-Fc" in Example 3 hereinafter.

Figure 15 shows the nucleotide and amino acid sequences (SEQ ID NOS: 19 and 20, respectively) of the molecule identified as "EMP-EMP-Fc" in Example 3 hereinafter.

Figure 16 shows the nucleotide and amino acid sequences (SEQ ID NOS: 21 and 22, respectively) of the molecule identified as "Fc-EMP-EMP" in Example 3 hereinafter.

Figures 17A and 17B show the DNA sequence (SEQ ID NO: 23) inserted into pCFM1656 between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites to form expression plasmid pAMG21 (ATCC accession no. 98113).

Figure 18A shows the hemoglobin, red blood cells, and hematocrit generated in vivo in normal female BDF1 mice treated with one 100 µg/kg bolus injection of various compounds. Figure 18B shows the same results with mice treated with 100 µg/kg per day delivered the same dose by 7-day micro-osmotic pump with the EMPs delivered at 100 µg/kg, rhEPO at 30U/mouse. (In both experiments, neutrophils, lymphocytes, and platelets were unaffected.) In these figures, the terms are defined as follows.

Fc-EMP: the compound of SEQ ID NO: 16 (Figure 13) dimerized with an identical second monomer (i.e., Cys residues 7 and 10 are bound to the corresponding Cys residues in the second monomer to form a dimer, as shown in Figure 2);

EMP-Fc: the compound of SEQ ID NO: 18 (Figure 14) dimerized in the same way as Fc-EMP except that the Fc domain is attached at

the C-terminal end rather than the N-terminal end of the EMP peptide.

“EMP-EMP-Fc” refers to a tandem repeat of the same peptide (SEQ ID NO: 20) attached to the same Fc domain by the carboxyl terminus of the peptides. “Fc-EMP-EMP” refers to the same tandem repeat of the peptide but with the same Fc domain attached at the amino terminus of the tandem repeat. All molecules are expressed in *E. coli* and so are not glycosylated.

Figures 19A and 19B show the nucleotide and amino acid sequences (SEQ ID NOS: 1055 and 1056) of the Fc-TNF- $\alpha$  inhibitor fusion molecule described in Example 4 hereinafter.

Figures 20A and 20B show the nucleotide and amino acid sequences (SEQ ID NOS: 1057 and 1058) of the TNF- $\alpha$  inhibitor-Fc fusion molecule described in Example 4 hereinafter.

Figures 21A and 21B show the nucleotide and amino acid sequences (SEQ ID NOS: 1059 and 1060) of the Fc-IL-1 antagonist fusion molecule described in Example 5 hereinafter.

Figures 22A and 22B show the nucleotide and amino acid sequences (SEQ ID NOS: 1061 and 1062) of the IL-1 antagonist-Fc fusion molecule described in Example 5 hereinafter.

Figures 23A, 23B, and 23C show the nucleotide and amino acid sequences (SEQ ID NOS: 1063 and 1064) of the Fc-VEGF antagonist fusion molecule described in Example 6 hereinafter.

Figures 24A and 24B show the nucleotide and amino acid sequences (SEQ ID NOS: 1065 and 1066) of the VEGF antagonist-Fc fusion molecule described in Example 6 hereinafter.

Figures 25A and 25B show the nucleotide and amino acid sequences (SEQ ID NOS: 1067 and 1068) of the Fc-MMP inhibitor fusion molecule described in Example 7 hereinafter.



Figures 26A and 26B show the nucleotide and amino acid sequences (SEQ ID NOS: 1069 and 1070) of the MMP inhibitor-Fc fusion molecule described in Example 7 hereinafter.

### Detailed Description of the Invention

#### 5           Definition of Terms

The terms used throughout this specification are defined as follows, unless otherwise limited in specific instances.

The term "comprising" means that a compound may include additional amino acids on either or both of the N- or C- termini of the  
10       given sequence. Of course, these additional amino acids should not significantly interfere with the activity of the compound.

The term "vehicle" refers to a molecule that prevents degradation and/or increases half-life, reduces toxicity, reduces immunogenicity, or increases biological activity of a therapeutic protein. Exemplary vehicles  
15       include an Fc domain (which is preferred) as well as a linear polymer (e.g., polyethylene glycol (PEG), polylysine, dextran, etc.); a branched-chain polymer (see, for example, U.S. Patent No. 4,289,872 to Denckenwalter et al., issued September 15, 1981; 5,229,490 to Tam, issued July 20, 1993; WO 93/21259 by Frechet et al., published 28 October 1993); a lipid; a  
20       cholesterol group (such as a steroid); a carbohydrate or oligosaccharide; or any natural or synthetic protein, polypeptide or peptide that binds to a salvage receptor. Vehicles are further described hereinafter.

The term "native Fc" refers to molecule or sequence comprising the sequence of a non-antigen-binding fragment resulting from digestion of  
25       whole antibody, whether in monomeric or multimeric form. The original immunoglobulin source of the native Fc is preferably of human origin and may be any of the immunoglobulins, although IgG1 and IgG2 are preferred. Native Fc's are made up of monomeric polypeptides that may be linked into dimeric or multimeric forms by covalent (i.e., disulfide

bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, IgGA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG (see Ellison et al. (1982), Nucleic Acids Res. 10: 4071-9). The term "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

The term "Fc variant" refers to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn. International applications WO 97/34631 (published 25 September 1997) and WO 96/32478 describe exemplary Fc variants, as well as interaction with the salvage receptor, and are hereby incorporated by reference. Thus, the term "Fc variant" comprises a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises sites that may be removed because they provide structural features or biological activity that are not required for the fusion molecules of the present invention. Thus, the term "Fc variant" comprises a molecule or sequence that lacks one or more native Fc sites or residues that affect or are involved in (1) disulfide bond formation, (2) incompatibility with a selected host cell (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody-dependent cellular cytotoxicity (ADCC). Fc variants are described in further detail hereinafter.

The term "Fc domain" encompasses native Fc and Fc variant molecules and sequences as defined above. As with Fc variants and native Fc's, the term "Fc domain" includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by other means.

The term "multimer" as applied to Fc domains or molecules comprising Fc domains refers to molecules having two or more polypeptide chains associated covalently, noncovalently, or by both covalent and non-covalent interactions. IgG molecules typically form dimers; IgM, pentamers; IgD, dimers; and IgA, monomers, dimers, trimers, or tetramers. Multimers may be formed by exploiting the sequence and resulting activity of the native Ig source of the Fc or by derivatizing (as defined below) such a native Fc.

The term "dimer" as applied to Fc domains or molecules comprising Fc domains refers to molecules having two polypeptide chains associated covalently or non-covalently. Thus, exemplary dimers within the scope of this invention are as shown in Figure 2.

The terms "derivatizing" and "derivative" or "derivatized" comprise processes and resulting compounds respectively in which (1) the compound has a cyclic portion; for example, cross-linking between cysteinyl residues within the compound; (2) the compound is cross-linked or has a cross-linking site; for example, the compound has a cysteinyl residue and thus forms cross-linked dimers in culture or in vivo; (3) one or more peptidyl linkage is replaced by a non-peptidyl linkage; (4) the N-terminus is replaced by  $-NRR^1$ ,  $-NRC(O)R^1$ ,  $-NRC(O)OR^1$ ,  $-NRS(O)_2R^1$ ,  $-NHC(O)NHR$ , a succinimide group, or substituted or unsubstituted benzyloxycarbonyl-NH-, wherein R and  $R^1$  and the ring substituents are as defined hereinafter; (5) the C-terminus is replaced by  $-C(O)R^2$  or  $-NR^3R^4$  wherein  $R^2$ ,  $R^3$  and  $R^4$  are as defined hereinafter; and (6) compounds in which individual amino acid moieties are modified through treatment with agents capable of reacting with selected side chains or terminal residues. Derivatives are further described hereinafter.

The term "peptide" refers to molecules of 2 to 40 amino acids, with molecules of 3 to 20 amino acids preferred and those of 6 to 15 amino acids

most preferred. Exemplary peptides may be randomly generated by any of the methods cited above, carried in a peptide library (e.g., a phage display library), or derived by digestion of proteins.

The term "randomized" as used to refer to peptide sequences refers to fully random sequences (e.g., selected by phage display methods) and sequences in which one or more residues of a naturally occurring molecule is replaced by an amino acid residue not appearing in that position in the naturally occurring molecule. Exemplary methods for identifying peptide sequences include phage display, *E. coli* display, ribosome display, RNA-peptide screening, chemical screening, and the like.

The term "pharmacologically active" means that a substance so described is determined to have activity that affects a medical parameter (e.g., blood pressure, blood cell count, cholesterol level) or disease state (e.g., cancer, autoimmune disorders). Thus, pharmacologically active peptides comprise agonistic or mimetic and antagonistic peptides as defined below.

The terms "-mimetic peptide" and "-agonist peptide" refer to a peptide having biological activity comparable to a protein (e.g., EPO, TPO, G-CSF) that interacts with a protein of interest. These terms further include peptides that indirectly mimic the activity of a protein of interest, such as by potentiating the effects of the natural ligand of the protein of interest; see, for example, the G-CSF-mimetic peptides listed in Tables 2 and 7. Thus, the term "EPO-mimetic peptide" comprises any peptides that can be identified or derived as described in Wrighton *et al.* (1996), *Science* 273: 458-63, Naranda *et al.* (1999), *Proc. Natl. Acad. Sci. USA* 96: 7569-74, or any other reference in Table 2 identified as having EPO-mimetic subject matter. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed

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therein by following the disclosed procedures with different peptide libraries.

The term "TPO-mimetic peptide" comprises peptides that can be identified or derived as described in Cwirla et al. (1997), Science 276: 1696-9, U.S. Pat. Nos. 5,869,451 and 5,932,946 and any other reference in Table 2 identified as having TPO-mimetic subject matter, as well as the U.S. patent application, "Thrombopoietic Compounds," filed on even date herewith and hereby incorporated by reference. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

The term "G-CSF-mimetic peptide" comprises any peptides that can be identified or described in Paukovits et al. (1984), Hoppe-Seylers Z. Physiol. Chem. 365: 303-11 or any of the references in Table 2 identified as having G-CSF-mimetic subject matter. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

The term "CTLA4-mimetic peptide" comprises any peptides that can be identified or derived as described in Fukumoto et al. (1998), Nature Biotech. 16: 267-70. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

The term "-antagonist peptide" or "inhibitor peptide" refers to a peptide that blocks or in some way interferes with the biological activity of the associated protein of interest, or has biological activity comparable to a known antagonist or inhibitor of the associated protein of interest. Thus, the term "TNF-antagonist peptide" comprises peptides that can be

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- identified or derived as described in Takasaki et al. (1997), Nature Biotech. 15: 1266-70 or any of the references in Table 2 identified as having TNF-antagonistic subject matter. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than
- 5 actually disclosed therein by following the disclosed procedures with different peptide libraries.

- The terms "IL-1 antagonist" and "IL-1ra-mimetic peptide" comprises peptides that inhibit or down-regulate activation of the IL-1 receptor by IL-1. IL-1 receptor activation results from formation of a
- 10 complex among IL-1, IL-1 receptor, and IL-1 receptor accessory protein. IL-1 antagonist or IL-1ra-mimetic peptides bind to IL-1, IL-1 receptor, or IL-1 receptor accessory protein and obstruct complex formation among any two or three components of the complex. Exemplary IL-1 antagonist or IL-1ra-mimetic peptides can be identified or derived as described in
- 15 U.S. Pat. Nos. 5,608,035, 5,786,331, 5,880,096, or any of the references in Table 2 identified as having IL-1ra-mimetic or IL-1 antagonistic subject matter. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide
- 20 libraries.

- The term "VEGF-antagonist peptide" comprises peptides that can be identified or derived as described in Fairbrother (1998), Biochem. 37: 17754-64, and in any of the references in Table 2 identified as having VEGF-antagonistic subject matter. Those of ordinary skill in the art
- 25 appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with different peptide libraries.

The term "MMP inhibitor peptide" comprises peptides that can be identified or derived as described in Koivunen (1999), Nature Biotech. 17:

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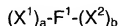
768-74 and in any of the references in Table 2 identified as having MMP inhibitory subject matter. Those of ordinary skill in the art appreciate that each of these references enables one to select different peptides than actually disclosed therein by following the disclosed procedures with  
5 different peptide libraries.

Additionally, physiologically acceptable salts of the compounds of this invention are also encompassed herein. By "physiologically acceptable salts" is meant any salts that are known or later discovered to be pharmaceutically acceptable. Some specific examples are: acetate;  
10 trifluoroacetate; hydrohalides, such as hydrochloride and hydrobromide; sulfate; citrate; tartrate; glycolate; and oxalate.

#### Structure of compounds

In General. In the compositions of matter prepared in accordance with this invention, the peptide may be attached to the vehicle through the  
15 peptide's N-terminus or C-terminus. Thus, the vehicle-peptide molecules of this invention may be described by the following formula I:

I



wherein:

20  $F^1$  is a vehicle (preferably an Fc domain);

$X^1$  and  $X^2$  are each independently selected from  $-(L^1)_c-P^1$ ,  $-(L^1)_c-P^1-(L^2)_d-P^2$ ,  $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$ , and  $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$

$P^1$ ,  $P^2$ ,  $P^3$ , and  $P^4$  are each independently sequences of pharmacologically active peptides;

25  $L^1$ ,  $L^2$ ,  $L^3$ , and  $L^4$  are each independently linkers; and

$a$ ,  $b$ ,  $c$ ,  $d$ ,  $e$ , and  $f$  are each independently 0 or 1, provided that at least one of  $a$  and  $b$  is 1.

Thus, compound I comprises preferred compounds of the formulae

II

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and multimers thereof wherein  $F^1$  is an Fc domain and is attached at the C-terminus of  $X^1$ ;

III



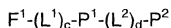
and multimers thereof wherein  $F^1$  is an Fc domain and is attached at the N-terminus of  $X^2$ ;

IV



10 and multimers thereof wherein  $F^1$  is an Fc domain and is attached at the N-terminus of  $-(L^1)_c-P^1$ ; and

V



15 and multimers thereof wherein  $F^1$  is an Fc domain and is attached at the N-terminus of  $-L^1-P^1-L^2-P^2$ .

Peptides. Any number of peptides may be used in conjunction with the present invention. Of particular interest are peptides that mimic the activity of EPO, TPO, growth hormone, G-CSF, GM-CSF, IL-1ra, leptin, CTLA4, TRAIL, TGF- $\alpha$ , and TGF- $\beta$ . Peptide antagonists are also of  
20 interest, particularly those antagonistic to the activity of TNF, leptin, any of the interleukins (IL-1, 2, 3, ...), and proteins involved in complement activation (e.g., C3b). Targeting peptides are also of interest, including tumor-homing peptides, membrane-transporting peptides, and the like. All of these classes of peptides may be discovered by methods described in  
25 the references cited in this specification and other references.

Phage display, in particular, is useful in generating peptides for use in the present invention. It has been stated that affinity selection from libraries of random peptides can be used to identify peptide ligands for any site of any gene product. Dedman *et al.* (1993), *J. Biol. Chem.* 268:



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23025-30. Phage display is particularly well suited for identifying peptides that bind to such proteins of interest as cell surface receptors or any proteins having linear epitopes. Wilson et al. (1998), Can. J. Microbiol. 44: 313-29; Kay et al. (1998), Drug Disc. Today 3: 370-8. Such proteins are  
5 extensively reviewed in Herz et al. (1997), J. Receptor & Signal Transduction Res. 17(5): 671-776, which is hereby incorporated by reference. Such proteins of interest are preferred for use in this invention.

A particularly preferred group of peptides are those that bind to cytokine receptors. Cytokines have recently been classified according to  
10 their receptor code. See Ingham (1997), Archivum Immunologiae et Therapiae Experimentalis 45: 353-7, which is hereby incorporated by reference. Among these receptors, most preferred are the CKRs (family I in Table 3). The receptor classification appears in Table 3.

15

Table 3—Cytokine Receptors Classified by Receptor Code

Cytokines (ligands)		Receptor Type	
family	subfamily	family	subfamily
I. Hematopoietic cytokines	1. IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 2. IL-3, IL-5, GM-CSF 3. IL-6, IL-11, IL-12, LIF, OSM, CNTF, leptin (OB) 4. G-CSF, EPO, TPO, PRL, GH 5. IL-17, HVS-IL-17	I. Cytokine R (CKR)	1. shared $\gamma$ Cr 2. shared GP 140 $\beta$ R 3. shared RP 130 4. "single chain" R 5. other R <sup>c</sup>
II. IL-10 ligands	IL-10, BCRF-1, HSV-IL-10	II. IL-10 R	
III. Interferons	1. IFN- $\alpha$ , $\alpha$ 2, $\alpha$ 4, m, t, IFN- $\beta$ <sup>d</sup> 2. IFN- $\gamma$	III. Interferon R	1. IFNAR 2. IFNGR
IV. IL-1 ligands	1. IL-1 $\alpha$ , IL-1 $\beta$ , IL-1Ra	IV. IL-1R	
V. TNF ligands	1. TNF- $\alpha$ , TNF- $\beta$ (LT), FAS1, CD40 L, CD30L, CD27 L	V. NGF/TNF R <sup>e</sup>	
VI. Chemokines	1. $\alpha$ chemokines: IL-8, GRO $\alpha$ , $\beta$ , $\gamma$ , IF-10, PF-4, SDF-1 2. $\beta$ chemokines: MIP1 $\alpha$ , MIP1 $\beta$ , MCP-1, 2, 3, 4, RANTES, eotaxin 3. $\gamma$ chemokines: lymphotactin	VI. Chemokine R	1. CXCR 2. CCR 3. CR 4. DARC <sup>f</sup>

<sup>c</sup> IL-17R belongs to the CKR family but is not assigned to any of the 4 indicated subfamilies.

<sup>d</sup> Other IFN type I subtypes remain unassigned. Hematopoietic cytokines, IL-10 ligands and interferons do not possess functional intrinsic protein kinases. The signaling molecules for the cytokines are JAK's, STATs and related non-receptor molecules. IL-14, IL-16 and IL-18 have been cloned but according to the receptor code they remain unassigned.

<sup>e</sup> TNF receptors use multiple, distinct intracellular molecules for signal transduction including "death domain" of FAS R and 55 kDa TNF- $\alpha$ R that participates in their cytotoxic effects. NGF/TNF R can bind both NGF and related factors as well as TNF ligands. Chemokine receptors are G protein-coupled, seven transmembrane (7TM, serpentine) domain receptors.

<sup>f</sup> The Duffy blood group antigen (DARC) is an erythrocyte receptor that can bind several different chemokines. It belongs to the immunoglobulin superfamily but characteristics of its signal transduction events remain unclear.

<p>VII. Growth factors</p> <p>1.1 SCF, M-CSF, PDGF-AA, AB, BB, FLT-3L, VEGF, SSV-PDGF</p> <p>1.2 FGF<math>\alpha</math>, FGF<math>\beta</math></p> <p>1.3 EGF, TGF-<math>\alpha</math>, VV-F19 (EGF-like)</p> <p>1.4 IGF-I, IGF-II, Insulin</p> <p>1.5 NGF, BDNF, NT-3, NT-4<sup>g</sup></p> <p>2. TGF-<math>\beta</math>1,<math>\beta</math>2,<math>\beta</math>3</p>	<p>VII. RKF</p> <p>1. TK sub-family</p> <p>1.1 IgTK III R</p> <p>1.2 IgTK IV R</p> <p>1.3 Cysteine-rich TK-I</p> <p>1.4 Cysteine rich TK-II</p> <p>1.5 Cysteine knot TK V</p> <p>2. STK subfamily<sup>h</sup></p>
--	---

Exemplary peptides for this invention appear in Tables 4 through 20 below. These peptides may be prepared by methods disclosed in the art. Single letter amino acid abbreviations are used. The X in these sequences (and throughout this specification, unless specified otherwise in a particular instance) means that any of the 20 naturally occurring amino acid residues may be present. Any of these peptides may be linked in tandem (i.e., sequentially), with or without linkers, and a few tandem-linked examples are provided in the table. Linkers are listed as "A" and may be any of the linkers described herein. Tandem repeats and linkers are shown separated by dashes for clarity. Any peptide containing a cysteinyl residue may be cross-linked with another Cys-containing peptide, either or both of which may be linked to a vehicle. A few cross-linked examples are provided in the table. Any peptide having more than one Cys residue may form an intrapeptide disulfide bond, as well; see, for example, EPO-mimetic peptides in Table 5. A few examples of intrapeptide disulfide-bonded peptides are specified in the table. Any of these peptides may be derivatized as described herein, and a few derivatized examples are provided in the table. Derivatized peptides in

<sup>g</sup> The neurotrophic cytokines can associate with NGF/TNF receptors also.

<sup>h</sup> STKS may encompass many other TGF- $\beta$ -related factors that remain unassigned. The protein kinases are intrinsic part of the intracellular domain of receptor kinase family (RKF). The enzymes participate in the signals transmission via the receptors.

the tables are exemplary rather than limiting, as the associated underivatized peptides may be employed in this invention, as well. For derivatives in which the carboxyl terminus may be capped with an amino group, the capping amino group is shown as  $-NH_2$ . For derivatives in

5 which amino acid residues are substituted by moieties other than amino acid residues, the substitutions are denoted by  $\sigma$ , which signifies any of the moieties described in Bhatnagar *et al.* (1996), *J. Med. Chem.* 39: 3814-9 and Cuthbertson *et al.* (1997), *J. Med. Chem.* 40: 2876-82, which are incorporated by reference. The J substituent and the Z substituents ( $Z_\sigma$ ,  $Z_\omega$ ,

10  $\dots Z_m$ ) are as defined in U.S. Pat. Nos. 5,608,035, 5,786,331, and 5,880,096, which are incorporated by reference. For the EPO-mimetic sequences (Table 5), the substituents  $X_1$  through  $X_{11}$  and the integer "n" are as defined in WO 96/40772, which is incorporated by reference. The substituents " $\Psi$ ," " $\Theta$ ," and "+" are as defined in Sparks *et al.* (1996), *Proc. Natl. Acad. Sci.* 93:

15 1540-4, which is hereby incorporated by reference.  $X_4$ ,  $X_5$ ,  $X_6$ , and  $X_7$  are as defined in U.S. Pat. No. 5,773,569, which is hereby incorporated by reference, except that: for integrin-binding peptides,  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ ,  $X_6$ ,  $X_7$ , and  $X_8$  are as defined in International applications WO 95/14714, published June 1, 1995 and WO 97/08203, published March 6, 1997, which

20 are also incorporated by reference; and for VIP-mimetic peptides,  $X_1$ ,  $X_1'$ ,  $X_1''$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ ,  $X_6$  and Z and the integers m and n are as defined in WO 97/40070, published October 30, 1997, which is also incorporated by reference. Xaa and Yaa below are as defined in WO 98/09985, published March 12, 1998, which is incorporated by reference.  $AA_1$ ,  $AA_2$ ,  $AB_1$ ,  $AB_2$ ,

25 and AC are as defined in International application WO 98/53842, published December 3, 1998, which is incorporated by reference.  $X^1$ ,  $X^2$ ,  $X^3$ , and  $X^4$  in Table 17 only are as defined in European application EP 0 911 393, published April 28, 1999. Residues appearing in boldface are D-amino acids. All peptides are linked through peptide bonds unless

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otherwise noted. Abbreviations are listed at the end of this specification. In the "SEQ ID NO." column, "NR" means that no sequence listing is required for the given sequence.

5

**Table 4—IL-1 antagonist peptide sequences**

Sequence/structure	SEQ ID NO:
Z <sub>11</sub> Z <sub>7</sub> Z <sub>8</sub> QZ <sub>5</sub> YZ <sub>6</sub> Z <sub>9</sub> Z <sub>10</sub>	212
XXQZ <sub>5</sub> YZ <sub>6</sub> XX	907
Z <sub>7</sub> ·XQZ <sub>5</sub> YZ <sub>6</sub> XX	908
Z <sub>7</sub> Z <sub>8</sub> QZ <sub>5</sub> YZ <sub>6</sub> Z <sub>9</sub> Z <sub>10</sub>	909
Z <sub>11</sub> Z <sub>7</sub> Z <sub>8</sub> QZ <sub>5</sub> YZ <sub>6</sub> Z <sub>9</sub> Z <sub>10</sub>	910
Z <sub>12</sub> Z <sub>13</sub> Z <sub>14</sub> Z <sub>15</sub> Z <sub>16</sub> Z <sub>17</sub> Z <sub>18</sub> Z <sub>19</sub> Z <sub>20</sub> Z <sub>21</sub> Z <sub>22</sub> Z <sub>11</sub> Z <sub>7</sub> Z <sub>8</sub> QZ <sub>5</sub> YZ <sub>6</sub> Z <sub>9</sub> Z <sub>10</sub> L	917
Z <sub>23</sub> NZ <sub>24</sub> Z <sub>25</sub> Z <sub>26</sub> Z <sub>27</sub> Z <sub>28</sub> Z <sub>29</sub> Z <sub>30</sub> Z <sub>40</sub>	979
TANVSSFWEWTPYYWQPYALPL	213
SWTDYGYWQPYALPISGL	214
ETPFTWEESNAYYWQPYALPL	215
ENTYSPNWADSMYWQPYALPL	216
SVGEDHNFWTSEYWQPYALPL	217
DGYDRWROSGERYWQPYALPL	218
FEWTPGYWQPY	219
FEWTPGYWQH	220
FEWTPGWYQJY	221
AcFEWTPGWYQJY	222
FEWTPGWpYQJY	223
FAWTPGYWQJY	224
FEWAPGYWQJY	225
FEWVPGYWQJY	226
FEWTPGYWQJY	227
AcFEWTPGYWQJY	228
FEWTPaWYQJY	229
FEWTPSarWYQJY	230
FEWTPGYYPY	231
FEWTPGWWQPY	232
FEWTPNYWQPY	233
FEWTPvYWQJY	234
FEWTPecGYWQJY	235
FEWTPAibYWQJY	236
FEWTPSarGYWQJY	237
FEWTPGYWQPY	238
FEWTPGYWQH	239
FEWTPGWYQJY	240
AcFEWTPGWYQJY	241
FEWTPGW-pY-QJY	242
FAWTPGYWQJY	243

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FEWAPGYWQJY	244
FEWVPGYWQJY	245
FEWTPGYWQJY	246
AcFEWTPGYWQJY	247
FEWTPAWYQJY	248
FEWTPSarWYQJY	249
FEWTPGYYPY	250
FEWTPGWWQPY	251
FEWTPNYWQPY	252
FEWTPVYWQJY	253
FEWTPecGYWQJY	254
FEWTPAibYWQJY	255
FEWTSarGYWQJY	256
FEWTPGYWOPYALPL	257
1NapEWTPGYQJY	258
YEWTPGYQJY	259
FEWVPGYYQJY	260
FEWTPSYQJY	261
FEWTPNYQJY	262
TKPR	263
RKSSK	264
RKQDK	265
NRKQDK	266
RKQDKR	267
ENRKQDKRF	268
VTKFYF	269
VTKFY	270
VTDFY	271
SHLYWQPYSVQ	671
TLVYWQPYSLQT	672
RGDYWQPYSVQS	673
VHVVWQPYSVQT	674
RLVYWQPYSVQT	675
SRVWFQPYSLQS	676
NMVYWQPYSIQT	677
SVVFWQPYSVQT	678
TFVYWQPYALPL	679
TLVYWQPYSIQR	680
RLVYWQPYSVQR	681
SPVFWQPYSIQI	682
WIEWWQPYSVQS	683
SLIYWQPYSLQM	684
TRLYWQPYSVQR	685
RCDYWQPYSVQT	686
MRVFWQPYSVQN	687
KIVYWQPYSVQT	688
RHLYWQPYSVQR	689
ALVWVWQPYSEOI	690
SRVWFQPYSLQS	691
WEQPYALPLE	692

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QLVWWQPYSVQR	693
DLRYWQPYSVQV	694
ELVWWQPYSLQL	695
DLVWWQPYSVQW	696
NGNYWQPYSFQV	697
ELVYWQPYSIQV	698
ELMYWQPYSVQE	699
NLLYWQPYSMQD	700
GYESWQPYSVQR	701
SRVWYQPYSVQR	702
LSEQYQPYSVQR	703
GGGWQPYSVQR	704
VGRWYQPYSVQR	705
VHVYWQPYSVQR	706
QARWYQPYSVQR	707
VHVYWQPYSVQT	708
RSVWYQPYSVQR	709
TRVWFQPYSVQR	710
GRIWFQPYSVQR	711
GRVWFQPYSVQR	712
ARTWYQPYSVQR	713
ARVWWQPYSVQM	714
RLMFYQPYSVQR	715
ESMWYQPYSVQR	716
HFGWWQPYSVHM	717
ARFWWQPYSVQR	718
RLVYWQ PYAPIY	719
RLVYWQ PYSYOT	720
RLVYWQ PYSLPI	721
RLVYWQ PYSVQA	722
SRVWYQ PYAKGL	723
SRVWYQ PYAQGL	724
SRVWYQ PYAMPL	725
SRVWYQ PYSVQA	726
SRVWYQ PYSLGL	727
SRVWYQ PYAREL	728
SRVWYQ PYSRQP	729
SRVWYQ PYFVQP	730
EYEWYQ PYALPL	731
IPEYWQ PYALPL	732
SRIWWQ PYALPL	733
DPLFWQ PYALPL	734
SRQWVQ PYALPL	735
IRSWWQ PYALPL	736
RGYWQ PYALPL	737
RLWVQ PYALPL	738
EYRWFQ PYALPL	739
DAYWVQ PYALPL	740
WSGYFQ PYALPL	741
NIEFWQ PYALPL	742

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TRDWVQ PYALPL	743
DSSWYQ PYALPL	744
IGNWYQ PYALPL	745
NLRWDQ PYALPL	746
LPEFWQ PYALPL	747
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TFVYWQPY SVQMTITGKVTM	764
TFVYWQPY SSHXXVPXGFPL	765
TFVYWQPY YGNPQWAIHVRH	766
TFVYWQPY VLLELPEGAVRA	767
TFVYWQPY VDYVWPIPIAQV	768
GWYQPYVDGWR	769
RWEQPYVKDGWS	770
EWYQPYALGWAR	771
GWYQPYARGL	772
LFEQPYAKALGL	773
GWEQPYARGLAG	774
AWVQPYATPLDE	775
MWYQPYSSOPAE	776
GWYQPYSSQGEV	777
DWFQPYSIQSDE	778
PWYQPYARGFG	779
RPLYWQPYSVQV	780
TLIYWQPYSVQI	781
RFDYWQPYSDQT	782
WHQFVQPYALPL	783
EWDS VYWQPYSVQ TLLR	784
WEQN VYWQPYSVQ SFAD	785
SDV VYWQPYSVQ SLEM	786
YYDG VYWQPYSVQ VMFA	787
SDIWWQ PYALPL	788
QRIWWQ PYALPL	789
SRIWWQ PYALPL	790
RSLYWQ PYALPL	791
TIWEQ PYALPL	792



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SYDWEQ PYALPL	794
SRIWCQ PYALPL	795
EIMFWQ PYALPL	796
DYVWQ PYALPL	797
MDLLVQ WYQPYALPL	798
GSKVIL WYQPYALPL	799
RQGANI WYQPYALPL	800
GGGDEP WYQPYALPL	801
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ETWVRE WYQPYALPL	803
KKGSTQ WYQPYALPL	804
LQARMN WYQPYALPL	805
EPRSOK WYQPYALPL	806
VKQKWR WYQPYALPL	807
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VWYWEQ PYALPL	814
ASEWWQ PYALPL	815
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VMEWWQ PYALPL	823
ERMWQ PYALPL	824
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WGNWYQ PYALPL	826
TLYWEQ PYALPL	827
VWRWEQ PYALPL	828
LLWTQ PYALPL	829
SRIWXX PYALPL	830
SDIWYQ PYALPL	831
WGYXX PYALPL	832
TSGWYQ PYALPL	833
VHPYXX PYALPL	834
EHSYFQ PYALPL	835
XXIWYQ PYALPL	836
AQLHSQ PYALPL	837
WANWFQ PYALPL	838
SRLYSQ PYALPL	839
GVTFSQ PYALPL	840
SIVWSQ PYALPL	841
SRDLVQ PYALPL	842

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SWHS VYWQPYSVQ SVPE	844
WRDS VYWQPYSVQ PESA	845
TWDA VYWQPYSVQ KWLD	846
TPPW VYWQPYSVQ SLDP	847
YWSS VYWQPYSVQ SVHS	848
YWW QPY ALGL	849
YWW QPY ALPL	850
EWI QPY ATGL	851
NWE QPY AKPL	852
AFY QPY ALPL	853
FLY QPY ALPL	854
VCK QPY LEWC	855
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QGWLTWQDSVOMYWQPYPALPL	857
FSEAGYTWPENTYWQPYPALPL	858
TESPGGLDWAKIYWQPYPALPL	859
DGYDRWRQSGERYWQPYPALPL	860
TANVSSFWEWTPGYWQPYPALPL	861
SVGEDHNFWTSE YWQPYPALPL	862
MNDQTSEVSTFP YWQPYPALPL	863
SWSEAFEQPRNL YWQPYPALPL	864
QYAEPSALNDWG YWQPYPALPL	865
NGDWATADWSNY YWQPYPALPL	866
THDEHI YWQPYPALPL	867
MLEKTYTWTWPG YWQPYPALPL	868
WSDPLTRDADL YWQPYPALPL	869
SDAFTTQDSQAM YWQPYPALPL	870
GDDAAWRDSDLT YWQPYPALPL	871
AIIRQLYRWSEM YWQPYPALPL	872
ENTYSPNWADSM YWQPYPALPL	873
MNDQTSEVSTFP YWQPYPALPL	874
SVGEDHNFWTSE YWQPYPALPL	875
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ENPFTWQESNAY YWQPYPALPL	877
VTPTWEDSNVF YWQPYPALPL	878
QIPFTWEQSNAY YWQPYPALPL	879
QAPLTWQESAAY YWQPYPALPL	880
EPTFTWEESKAT YWQPYPALPL	881
TTTLTWEESNAY YWQPYPALPL	882
ESPLTWEESNAL YWQPYPALPL	883
ETPLTWEESNAY YWQPYPALPL	884
EATFTWAEASNAY YWQPYPALPL	885
EALFTWKESTAY YWQPYPALPL	886
STP-TWEESNAY YWQPYPALPL	887
ETPFTWEESNAY YWQPYPALPL	888
KAPFTWEESQAY YWQPYPALPL	889
STSFTWEESNAY YWQPYPALPL	890
DSTFTWEESNAY YWQPYPALPL	891
YIPFTWEESNAY YWQPYPALPL	892

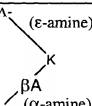

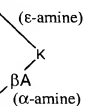

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ETLFTWEESNAT YWQPYALPL	894
VSSFTWEESNAY YWQPYALPL	895
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Py-1-NapPYQJYALPL	897
TANVSSFEWTPG YWQPYALPL	898
FEWTPGYWQPYALPL	899
FEWTPGYWQJYALPL	900
FEWTPGYQJYALPL	901
ETPFTWEESNAYYWQPYALPL	902
FTWEESNAYYQJYALPL	903
ADVL YWQPYA PVTLWV	904
GDVAE YWQPYA LPLTSL	905
SWTDYG YWQPYA LPISGL	906
FEWTPGYWQPYALPL	911
FEWTPGYWQJYALPL	912
FEWTPGWYQPYALPL	913
FEWTPGWYQJYALPL	914
FEWTPGYQPYALPL	915
FEWTPGYQJYALPL	916
TANVSSFEWTPGYWQPYALPL	918
SWTDYGYWQPYALPISGL	919
ETPFTWEESNAYYWQPYALPL	920
ENTYSPNWADSMYWQPYALPL	921
SVGEDHNFWTSEYWQPYALPL	922
DGYDRWRQSGERYWQPYALPL	923
FEWTPGYWQPYALPL	924
FEWTPGYWQPY	925
FEWTPGYWQJY	926
EWTPGYWQPY	927
FEWTPGWYQJY	928
AEWTPGYWQJY	929
FAWTPGYWQJY	930
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FEWAPGYWQJY	932
FEWTAGYWQJY	933
FEWTPAYWQJY	934
FEWTPGAWQJY	935
FEWTPGYAQJY	936
FEWTPGYWQJA	937
FEWTTGGYWQJY	938
FEWTPGYWQJY	939
FEWTJGYWQJY	940
FEWTPecGYWQJY	941
FEWTPAibYWQJY	942
FEWTPSarWYQJY	943
FEWTPSarGYWQJY	944
FEWTPNYWQJY	945
FEWTPVYWQJY	946

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FEWTVPYWQJY	947
AcFEWTPGWYQJY	948
AcFEWTPGYWQJY	949
INap-EWTPGYQJY	950
YEWTPGYQJY	951
FEWVPGYYQJY	952
FEWTPGYQJY	953
FEWTPsYYQJY	954
FEWTPnYYQJY	955
SHLY-Nap-QPYSVQM	956
TLVY-Nap-QPYSLOT	957
RGDY-Nap-QPYSVQS	958
NMVY-Nap-QPYSIQT	959
VYWQPYSVQ	960
VY-Nap-QPYSVQ	961
TFVYWQJYALPL	962
FEWTPGYQJ-Bpa	963
XaaFEWTPGYQJ-Bpa	964
FEWTPGY-Bpa-QJY	965
AcFEWTPGY-Bpa-QJY	966
FEWTPG-Bpa-YQJY	967
AcFEWTPG-Bpa-YQJY	968
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AcFE-Bpa-TPGYQJY	970
Bpa-EWTPGYQJY	971
AcBpa-EWTPGYQJY	972
VYWQPYSVQ	973
RLVYWQPYSVQR	974
RLVY-Nap-QPYSVQR	975
RLDYWQPYSVQR	976
RLVWFQPYSVQR	977
RLVYWQPYSIQR	978
DNSSWYDSFLL	980
DNTAWYESFLA	981
DNTAWYENFLL	982
PARE DNTAWYDSFLI WC	983
TSEY DNTTWYEFLLA SQ	984
SQIP DNTAWYQSFLH HG	985
SPFI DNTAWYENFLL TY	986
EQIY DNTAWYDHFL SY	987
TPFI DNTAWYENFLL TY	988
TYTY DNTAWYERFLM SY	989
TMTQ DNTAWYENFLL SY	990
TI DNTAWYANLVO TYPQ	991
TI DNTAWYERFLA QYPD	992
HI DNTAWYENFLL TYTP	993
SQ DNTAWYENFLL SYKA	994
QI DNTAWYERFL QYNA	995
NQ DNTAWYESFLL QYNT	996
TI DNTAWYENFLL NHNL	997

HY DNTAWYERFLO QGWH	998
ETPFTWEESNAYYWQPYALPL	999
YIPFTWEESNAYYWQPYALPL	1000
DGYDRWRQSGERYWQPYALPL	1001
pY-INap-pY-QJYALPL	1002
TANVSSFEWTPGYWQPYALPL	1003
FEWTPGYWQJYALPL	1004
FEWTPGYWQPYALPLSD	1005
FEWTPGYYQJYALPL	1006
FEWTPGYWQJY	1007
AcFEWTPGYWQJY	1008
AcFEWTPGWYQJY	1009
AcFEWTPGYYQJY	1010
AcFEWTPaYWQJY	1011
AcFEWTPaWYQJY	1012
AcFEWTPaYYQJY	1013
FEWTPGYYQJYALPL	1014
FEWTPGYWQJYALPL	1015
FEWTPGWYQJYALPL	1016
TANVSSFEWTPGYWQPYALPL	1017
AcFEWTPGYWQJY	1018
AcFEWTPGWYQJY	1019
AcFEWTPGYYQJY	1020
AcFEWTPAYWQJY	1021
AcFEWTPAWYQJY	1022
AcFEWTPAYYQJY	1023

Table 5—EPO-mimetic peptide sequences

Sequence/structure	SEQ ID NO:
YXCXXGPXTWXCXP	83
YXCXXGPXTWXCXP-YXCXXGPXTWXCXP	84
YXCXXGPXTWXCXP- $\Lambda$ -YXCXXGPXTWXCXP	85
YXCXXGPXTWXCXP- $\Lambda$ - 	86
YXCXXGPXTWXCXP- $\Lambda$ - 	86
GGTYSCHFGPLTWVCKPQGG	87
GGDYHCRMGPLTWVCKPLGG	88
GGVYACRMGPITWVCSPLGG	89
VGNMCHFGPITWVCRPGGG	90
GGLYLCRFGPVTWDCGYKGG	91
GGTYSCHFGPLTWVCKPQGG-	92
GGTYSCHFGPLTWVCKPQGG-	92
GGTYSCHFGPLTWVCKPQGG- $\Lambda$ -	93
GGTYSCHFGPLTWVCKPQGG	93
GGTYSCHFGPLTWVCKPQGGSSK	94
GGTYSCHFGPLTWVCKPQGGSSK-	95
GGTYSCHFGPLTWVCKPQGGSSK	95
GGTYSCHFGPLTWVCKPQGGSSK- $\Lambda$ -	96
GGTYSCHFGPLTWVCKPQGGSSK	96
GGTYSCHFGPLTWVCKPQGGSS- 	97
GGTYSCHFGPLTWVCKPQGGSS- 	97
GGTYSCHFGPLTWVCKPQGGSSK(- $\Lambda$ -biotin)	98
CX <sub>4</sub> X <sub>5</sub> GPX <sub>6</sub> TWX <sub>7</sub> C	421
GGTYSCHGPLTWVCKPQGG	422
VGNMAHMGPIWVCRPGG	423
GGPHHVYACRMGPLTWIC	424
GGTYSCHFGPLTWVCKPQ	425
GGLYACHMGPMTWVCCPLRG	426
TIAQYICYMGPEWECRPSKA	427
YSCHFGPLTWVCK	428
YCHFGPLTWVC	429
X <sub>3</sub> X <sub>4</sub> X <sub>5</sub> GPX <sub>6</sub> TWX <sub>7</sub> X <sub>8</sub>	124
YX <sub>2</sub> X <sub>3</sub> X <sub>4</sub> X <sub>5</sub> GPX <sub>6</sub> TWX <sub>7</sub> X <sub>8</sub>	461

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$X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$	419
$X_1YX_2CX_3X_5GPX_6TWX_7CX_9X_{10}X_{11}$	420
GGLYLCHFGPVTWDCGYKGG	1024
GGTYSCHFGPLTWVCKPQGG	1025
GGDYHCRMGPPLTWVCKPLGG	1026
VGNYMCHFGPITWVCRPGGG	1029
GGVYACRMGPITWVCSPLGG	1030
VGNYMAHMGPIWVCRPGG	1035
GGTYSCHFGPLTWVCKPQ	1036
GGLYACHMGPMPLTWVCPPLRG	1037
TIAQYICYMGPETWECRPSPKA	1038
YSCHFGPLTWVCK	1039
YCHFGPLTWVC	1040
SCHFGPLTWVCK	1041
$(AX_2)X_1X_3X_4X_5GPX_6TWX_7X_8$	1042

Table 6—TPO-mimetic peptide sequences

Sequence/structure	SEQ ID NO:
IEGPTLRQWLAARA	13
IEGPTLRQWLAAKA	24
IEGPTLREWLAARA	25
IEGPTLRQWLAARA- $\Lambda$ -IEGPTLRQWLAARA	26
IEGPTLRQWLAAKA- $\Lambda$ -IEGPTLRQWLAAKA	27
IEGPTLRQCLAARA- $\Lambda$ -IEGPTLRQCLAARA	28
IEGPTLRQWLAARA- $\Lambda$ -K(BrAc)- $\Lambda$ -IEGPTLRQWLAARA	29
IEGPTLRQWLAARA- $\Lambda$ -K(PEG)- $\Lambda$ -IEGPTLRQWLAARA	30
IEGPTLRQCLAARA- $\Lambda$ -IEGPTLRQWLAARA	31
IEGPTLRQCLAARA- $\Lambda$ -IEGPTLRQWLAARA	31
IEGPTLRQWLAARA- $\Lambda$ -IEGPTLRQCLAARA	32
IEGPTLRQWLAARA- $\Lambda$ -IEGPTLRQCLAARA	32
VRDQIXXL	33
TLREWL	34
GRVRDQVAGW	35
GRVKDQIAQL	36
GVRDQVSWAL	37
ESVREQVMKY	38
SVRSQISASL	39
GVRETVYRHM	40
GVREVVMHML	41
GRVRDQIWAL	42
AGVRDQILWL	43
GRVRDQIMLSL	44
GRVRDQ(X) <sub>2</sub> L	45
CTLROWLQGC	46
CTLQEFLEG	47
CTRTEWLHGC	48
CTLREWLHGGFC	49
CTLREWVFAGLC	50
CTLROWLILLGMC	51
CTLAEFLLASGVEQC	52
CSLQEFLLSHGGYVC	53
CTLREFLDPTTAVC	54
CTLKEWLVSHEVWC	55
CTLREWL(X) <sub>2-6</sub> C	56-60
REGPTLRQWM	61
EGPTLRQWLA	62
ERGPFWAKAC	63
REGPRCVMWM	64
CGTEGPTLSTWLDC	65



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CEQDGPTLLEWLKC	66
CELVGPSLMSWLTC	67
CLTGPFVTQWLYEC	68
CRAGPTLLEWLTLC	69
CADGPTLREWISFC	70
C(X) <sub>1,2</sub> EGPTLREWL(X) <sub>1,2</sub> C	71-74
GGCTLREWLHGGFCGG	75
GGCADGPTLREWISFCGG	76
GNADGPTLRQWLEGRRPKN	77
LAIEGPTLRQWLHGNGRDT	78
HGRVGPTLREWKTQVATKK	79
TIKGPTLRQWLKSREHTS	80
ISDGPTLKEWLSVTRGAS	81
SIEGPTLREWLTSRTPHS	82

Table 7—G-CSF-mimetic peptide sequences

Sequence/structure	SEQ ID NO:
EEDCK	99
EEDCK	99
EEDCK	99
EEDσK	100
EEDσK	100
EEDσK	100
pGluEDσK	101
pGluEDσK	101
pGluEDσK	101
PicSDσK	102
PicSDσK	102
PicSDσK	102
EEDCK-Λ-EEDCK	103
EEDXK-Λ-EEDXK	104

Table 8—TNF-antagonist peptide sequences

Sequence/structure	SEQ ID NO:
YCFTASENHCY	106
YCFTNSENHCY	107
YCFTRSENHCY	108
FCASENHCY	109
YCASENHCY	110
FCNSENHCY	111
FCNSENRCY	112
FCNSVENRCY	113
YCSQSVSND CF	114
FCVSNDR CY	115
YCRKELGQVCY	116
YCKEPGQCY	117
YCRKEMGCY	118
FCRKEMGCY	119
YCW SQNL CY	120
YCELSQYLCY	121
YCW SQNY CY	122
YCW SQYLCY	123
DFLPHYKNTSLGHRP	1085
$  \begin{array}{c}  \text{AA}_1\text{-AB}_1 \\  \quad \backslash \\  \quad \quad \text{AC} \\  \quad / \\  \text{AA}_2\text{-AB}_2  \end{array}  $	NR

**Table 9—Integrin-binding peptide sequences**

Sequence/structure	SEQ ID NO:
RX <sub>1</sub> ETX <sub>2</sub> WX <sub>3</sub>	441
RX <sub>1</sub> ETX <sub>2</sub> WX <sub>3</sub>	442
RGDGX	443
CRGDGXC	444
CX <sub>1</sub> X <sub>2</sub> RLDX <sub>3</sub> X <sub>4</sub> C	445
CARRLDAPC	446
CPSRLDSPC	447
X <sub>1</sub> X <sub>2</sub> X <sub>3</sub> RGDX <sub>4</sub> X <sub>5</sub> X <sub>6</sub>	448
CX <sub>2</sub> CRGDGX <sub>5</sub> C	449
CDCRGDCFC	450
CDCRGDCLC	451
CLCRGDCIC	452
X <sub>1</sub> X <sub>2</sub> DDX <sub>4</sub> X <sub>5</sub> X <sub>7</sub> X <sub>8</sub>	453
X <sub>1</sub> X <sub>2</sub> X <sub>3</sub> DDX <sub>4</sub> X <sub>5</sub> X <sub>6</sub> X <sub>7</sub> X <sub>8</sub>	454
CWDDGWLC	455
CWDDLWWLC	456
CWDDGLMC	457
CWDDGWMC	458
CSWDDGWLC	459
CPDDLWWLC	460
NGR	NR
GSL	NR
RGD	NR
CGRECPRLCQSSC	1071
CNGRCVSGCAGRC	1072
CLSGSLSC	1073
RGD	NR
NGR	NR
GSL	NR
NGRAHA	1074
CNGRC	1075
CDCRGDCFC	1076
CGSLVRC	1077
DLXXL	1043
RTDLSLRTYTL	1044
RTDLSLRTY	1053
RTDLSLRT	1054
RTDLSLR	1078
GDLLKLRLTL	1079
GDLSLRQLLSR	1080
RDDLHMLRLQLW	1081
SSDLHALKKRYG	1082
RGDLKQLSELTW	1083
RGDLAALSAPPV	1084

**Table 10—Selectin antagonist peptide sequences**

Sequence/structure	SEQ ID NO:
DITWDQLWDLMK	147
DITWDELWKIMN	148
DYTWFLWDMMQ	149
QITWAQLWNMMK	150
DMTWHDLWTLMS	151
DYSWHDLWEMMS	152
EITWDQLWEVMN	153
HVSWEQLWDIMN	154
HITWDQLWRIMT	155
RNMSWLLEWEHMK	156
AEWTWDQLWHVMNPAESQ	157
HRAEWLALWEQMSP	158
KKEDWLALWRIMSV	159
ITWDQLWDLMK	160
DITWDQLWDLMK	161
DITWDQLWDLMK	162
DITWDQLWDLMK	163
CQNRYTDLVAIQNKNE	462
AENWADNEPNNKRNNED	463
RKNNKTWTWVGTKKALTNE	464
KKALTNEAENWAD	465
CQXRYTDLVAIQNKXE	466
RKXNXXTWTWVGTXKXLTEE	467
AENWADGEPNNKXNXED	468
CXXXYTXXLVAIQNKXE	469
RKXXXXXXWVGTXKXLTXE	470
AXNXXXXEPNNXXED	471
XXKXKTXEAXNWXX	472

Table 11—Antipathogenic peptide sequences

Sequence/structure	SEQ ID NO:
GFFALIPKIISSPLFKTLLSAVGSALSSSGGQQ	503
GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE	504
GFFALIPKIISSPLFKTLLSAV	505
GFFALIPKIISSPLFKTLLSAV	506
KGFFALIPKIISSPLFKTLLSAV	507
KKGFFALIPKIISSPLFKTLLSAV	508
KKGFFALIPKIISSPLFKTLLSAV	509
GFFALIPKIIS	510
GIGAVLKVLTTGLPALISWIKRKRQQ	511
GIGAVLKVLTTGLPALISWIKRKRQQ	512
GIGAVLKVLTTGLPALISWIKRKRQQ	513
GIGAVLKVLTTGLPALISWIKR	514
AVLKVLTTGLPALISWIKR	515
KLLLLKLLLK	516
KLLLLKLLLK	517
KLLKLLKLLK	518
KLLKLLKLLK	519
KLLKLLKLLK	520
KLLKLLKLLK	521
KLLLK	522
KLLKLLK	523
KLLKLLKLLK	524
KLLKLLKLLK	525
KLLKLLKLLK	526
KAAAAAATAAK	527
KVVVKVVVKVK	528
KVVVKVVVKVK	529
KVVVKVVVKVK	530
KVVVKVVVKVK	531
KLILKL	532
KVLHLL	533
LKLALL	534
KPLHLL	535
KLILKLVK	536
KVFHLLH	537
HKFRILKL	538
KPFHILHL	539
KIIKIKIKIK	540
KIIKIKIKIK	541
KIIKIKIKIK	542
KIPIKIKIKIPK	543
KIPIKIKIKIVK	544
RIIRIRIRIR	545
RIIRIRIRIR	546
RIIRIRIRIR	547
RIVIRIRIRIR	548

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RIIVRIALRIIR	549
RIGIRLVRRIIR	550
KIVIRIRILRIIR	551
RIAVKWALRFIK	552
KIGWKLVRRIIR	553
KKIGWLIIRVRR	554
RIVIRIRILRIIR	555
RIIVRIALRIIRVR	556
RIGIRLVRRIIRRV	557
KIVIRIRARLIRIRIR	558
RIIVKIRLRIIKKIRL	559
KIGIKARVRIIRVKII	560
RIIVHIIRLRIIHHIRL	561
HIGIKAHVRIIRVHII	562
RIYVKIHLRYIKKIRL	563
KIGHKARVHIIRYKII	564
RIYVKPHPRYIKKIRL	565
KPGHKARPHIIRYKII	566
KIVIRIRILRIIRIRKIV	567
RIIVKIRLRIIKKIRLIK	568
KIGWKLVRRIIRVKIGALR	569
KIVIRIRILRIIRIRKIVVKRIIR	570
RFVAVKIRLRIIKKIRLIKIRKRVIK	571
KAGWKLVRRIIRVKIGALRIGWKKRVRIK	572
RIYVKPHPRYIKKIRL	573
KPGHKARPHIIRYKII	574
KIVIRIRILRIIRIRKIV	575
RIIVKIRLRIIKKIRLIK	576
RIYVSKISYIKKIRL	577
KIVIFTRIIRLTSIRIRISV	578
KPIHKARPTIIRYKMI	579
cyclicCKGGFFALIPKIISSPLFKTLLSAVC	580
CKKGFFALIPKIISSPLFKTLLSAVC	581
CKKKGGFFALIPKIISSPLFKTLLSAVC	582
CyclicCRIVIRIRILRIIRIC	583
CyclicCKPGHKARPHIIRYKIIIC	584
CyclicCRFAVKIRLRIIKKIRLIKIRKRVIKC	585
KLLLKLL KLLKC	586
KLLLKLLKLLK	587
KLLLKLLKLLKC	588
KLLLKLLKLLK	589

Table 12—VIP-mimetic peptide sequences

Sequence/structure	SEQ ID NO:
HSDAVFYDNYTR LRKQMAVKKYL N SILN	590
Nle HSDAVFYDNYTR LRKQMAVKKYL N SILN	591
X <sub>1</sub> X <sub>1</sub> ' X <sub>1</sub> " X <sub>2</sub>	592
X <sub>3</sub> S X <sub>4</sub> LN	593
NH CH CO KKYX5 NH CH CO X6	594
<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <math>\downarrow</math> (CH<sub>2</sub>)<sub>m</sub> </div> <div style="text-align: center;"> <math>\downarrow</math> (CH<sub>2</sub>)<sub>n</sub> </div> </div>	
KKYL	595
NSILN	596
KKYL	597
KKYA	598
AVKKYL	599
NSILN	600
KKYV	601
SILauN	602
KKYLNle	603
NSYLN	604
NSIYN	605
KKYLPNSILN	606
LauKKYL	607
CapKKYL	608
KYL	NR
KKYNle	609
VKKYL	610
LNSILN	611
YLNLSILN	612
KKYLN	613
KKYLN S	614
KKYLN SI	615
KKYLN SIL	616
KKYL	617
KKYDA	618
AVKKYL	619
NSILN	620
KKYV	621
SILauN	622
NSYLN	623
NSIYN	624
KKYLNle	625
KKYLPNSILN	626
KKYL	627
KKYDA	628
AVKKYL	629
NSILN	630
KKYV	631
SILauN	632



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LauKKYL	633
CapKKYL	634
KYL	NR
KYL	NR
KKYNle	635
VKKYL	636
LNSILN	637
YLNILN	638
KKYLNle	639
KKYLN	640
KKYLN	641
KKYLN	642
KKYLN	643
KKYLD	644
cyclicCKKYLC	645
CKKYLK	646
↓ S-CH <sub>2</sub> -CO	
KKYA	647
WWTDTGLW	648
WWTDDGLW	649
WWDTRGLWVWTI	650
FWGNDGIWLESG	651
DWDQFGLWRGAA	652
RWDDNGLWVVVL	653
SGMWSHYGIWMG	654
GGRWDQAGLWVA	655
KLWSEQGIWMGE	656
CWSMHGLWLC	657
GCWDNTGIWVPC	658
DWDTRGLWVY	659
SLWDENGAWI	660
KWDDRGLWMH	661
QAWNERGLWT	662
QWDTRGLWVA	663
WNVHGIWQE	664
SWDTRGLWVE	665
DWDTRGLWVA	666
SWGRDGLWIE	667
EWTDNGLWAL	668
SWDEKGLWSA	669
SWDSSGLWMD	670

Table 13—Mdm/hdm antagonist peptide sequences

Sequence/structure	SEQ ID NO:
TFSDLW	130
QETFSDLWKLLP	131
QPTFSDLWKLLP	132
QETFSDYWKLLP	133
QPTFSDYWKLLP	134
MPRFMDYWEGLN	135
VQNFIDYWTQQF	136
TGPAFTHYWATF	137
IDRAPTFRDHWFALV	138
PRPALVFADYWETLY	139
PAFSRFWSDSL SAGAH	140
PAFSRFWSKLSAGAH	141
PXFXDYWXXL	142
QETFSDLWKLLP	143
QPTFSDLWKLLP	144
QETFSDYWKLLP	145
QPTFSDYWKLLP	146

Table 14—Calmodulin antagonist peptide sequences

Sequence/structure	SEQ ID NO:
SCVKWGGKKEFCGS	164
SCWKYWGKECGS	165
SCYEWGKLRWCGS	166
SCLR WGWKWSNCGS	167
SCWRWGGKYQICGS	168
SCVSWGALKCGS	169
SCIRWGGQNTFCGS	170
SCWQWGNLKICGS	171
SCVRWGGQSLICGS	172
LKKFNARRKLKGAILTTMLAK	173
RRWKKNFIAVSAANRFKK	174
RKWQKTGHAVRAIGRLSS	175
INLKALAAALKIL	176
KIWSILAPLGTTLVKLVA	177
LKKLLKLKLLKL	178
LKWKLLKLKLLKLL	179
AEWPSLTEIKTLSHFVS	180
AEWPSPTRVISTTYFGS	181
AELAHWPPVKTVLRST	182
AEGSWLQLLNLMKOMNN	183
AEWPSLTEIK	184

**Table 15—Mast cell antagonists/Mast cell protease inhibitor  
peptide sequences**

<b>Sequence/structure</b>	<b>SEQ ID NO:</b>
SGSGVLKRPLPILPVTR	272
RWLSSRPLPLPLPPRT	273
GSGSYDTLALPSLPLHPMSS	274
GSGSYDTRALPSLPLHPMSS	275
GSGSSGVTMYPKLPPHWSMA	276
GSGSSGVRMYPKLPPHWSMA	277
GSGSSSMRMVPTIPGSAKHG	278
RNR	NR
QT	NR
RQK	NR
NRQ	NR
RQK	NR
RNRQKT	436
RNRQ	437
RNRQK	438
NRQKT	439
RQKT	440

Table 16—SH3 antagonist peptide sequences

Sequence/structure	SEQ ID NO:
RPLPLP	282
RELPLP	283
SPLPLP	284
GPLPLP	285
RPLPIP	286
RPLPIP	287
RRLPPTP	288
RQLPPTP	289
RPLPSRP	290
RPLTRP	291
SRLPLP	292
RALPSP	293
RRLPRT	294
RPVPPIT	295
ILAPPVP	296
RPLMLP	297
RPLPLP	298
RPLSLP	299
RPLSLP	300
RPLMIP	301
RPLPLP	302
RPLPPT	303
RSLPLP	304
RPQPPPP	305
RQLPIP	306
XXXRPLPLPXP	307
XXXRPLPIPXX	308
XXXRPLPLPXX	309
RXXRPLPLPXP	310
RXXRPLPLPPP	311
PPYPPPPPIPXX	312
PPYPPPPVPXX	313
LXXRPLPXP	314
ΨXXRPLPXL	315
PPXθXPPPVP	316
+PPΨPXKPXWL	317
RPX'Y'P'YR+SXP	318
PPVPPRPXTL	319
ΨPΨLPΨK	320
+θDXLPXLP	321

Table 17—Somatostatin or cortistatin mimetic peptide sequences

Sequence/structure	SEQ ID NO:
X <sup>1</sup> -X <sup>2</sup> -Asn-Phe-Phe-Trp-Lys-Thr-Phe-X <sup>3</sup> -Ser-X <sup>4</sup>	473
Asp Arg Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys Lys	474
Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys Lys	475
Cys Arg Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys Lys	476
Asp Arg Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys	477
Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys	478
Cys Arg Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys	479
Asp Arg Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys	480
Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys Lys	481
Cys Lys Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys Lys	482
Asp Arg Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys	483
Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys	484
Cys Lys Asn Phe Phe Trp Lys Thr Phe Ser Ser Cys	485
Asp Arg Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys Lys	486
Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys Lys	487
Cys Arg Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys Lys	488
Asp Arg Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys	489
Met Pro Cys Arg Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys	490
Cys Arg Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys	491
Asp Arg Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys Lys	492
Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys Lys	493
Cys Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys Lys	494
Asp Arg Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys	495
Met Pro Cys Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys	496
Cys Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys	497

Table 18—UKR antagonist peptide sequences

Sequence/structure	SEQ ID NO:
AEMPHSLNFSQYLWYT	196
AEHTYSSLWDYSPPLAF	197
AELDLWMRHYPPLSFSNR	198
AESSLWTRYAWPSPMSY	199
AEWHFGLSFGSYLWSKT	200
AEPALNWSFFFNPGLH	201
AEWSFYNLHLPPEQTIF	202
AEPLDLWSLYSLPPLAM	203
AEPTLWQLYQFPLRLSG	204
AEISFSELMWLRSTPAF	205
AELSEADLWTTWFGMGS	206
AESSLWRIFSPSALMMS	207
AESLPTLSILWGKESV	208
AETLFMDLWHDKHILLT	209
AEILNFPLWHEPLWSTE	210
AESQTGLNNTLFWNTLR	211
AEPVYQYELDSYLRSY	430
AELDLSTFYDIQYLLRT	431
AEFFKLGPNGYVYLHSA	432
FKLXXGYVYL	433
AESTYHHLSLGYMYTLN	434
YHXLXXGYMYT	435

**Table 19—Macrophage and/or  
T-cell inhibiting peptide sequences**

<b>Sequence/structure</b>	<b>SEQ ID NO:</b>
Xaa-Yaa-Arg	NR
Arg-Yaa-Xaa	NR
Xaa-Arg-Yaa	NR
Yaa-Arg-Xaa	NR
Ala-Arg	NR
Arg-Arg	NR
Asn-Arg	NR
Asp-Arg	NR
Cys-Arg	NR
Gln-Arg	NR
Glu-Arg	NR
Gly-Arg	NR
His-arg	NR
Ile-Arg	NR
Leu-Arg	NR
Lys-Arg	NR
Met-Arg	NR
Phe-Arg	NR
Ser-Arg	NR
Thr-Arg	NR
Trp-Arg	NR
Tyr-Arg	NR
Val-Arg	NR
Ala-Glu-Arg	NR
Arg-Glu-Arg	NR
Asn-Glu-Arg	NR
Asp-Glu-Arg	NR
Cys-Glu-Arg	NR
Gln-Glu-Arg	NR
Glu-Glu-Arg	NR
Gly-Glu-Arg	NR
His-Glu-Arg	NR
Ile-Glu-Arg	NR
Leu-Glu-Arg	NR
Lys-Glu-Arg	NR
Met-Glu-Arg	NR
Phe-Glu-Arg	NR
Pro-Glu-Arg	NR
Ser-Glu-Arg	NR
Thr-Glu-Arg	NR
Trp-Glu-Arg	NR
Tyr-Glu-Arg	NR
Val-Glu-Arg	NR

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Arg-Ala	NR
Arg-Asp	NR
Arg-Cys	NR
Arg-Gln	NR
Arg-Glu	NR
Arg-Gly	NR
Arg-His	NR
Arg-Ile	NR
Arg-Leu	NR
Arg-Lys	NR
Arg-Met	NR
Arg-Phe	NR
Arg-Pro	NR
Arg-Ser	NR
Arg-Thr	NR
Arg-Trp	NR
Arg-Tyr	NR
Arg-Val	NR
Arg-Glu-Ala	NR
Arg-Glu-Asn	NR
Arg-Glu-Asp	NR
Arg-Glu-Cys	NR
Arg-Glu-Gln	NR
Arg-Glu-Glu	NR
Arg-Glu-Gly	NR
Arg-Glu-His	NR
Arg-Glu-Ile	NR
Arg-Glu-Leu	NR
Arg-Glu-Lys	NR
Arg-Glu-Met	NR
Arg-Glu-Phe	NR
Arg-Glu-Pro	NR
Arg-Glu-Ser	NR
Arg-Glu-Thr	NR
Arg-Glu-Trp	NR
Arg-Glu-Tyr	NR
Arg-Glu-Val	NR
Ala-Arg-Glu	NR
Arg-Arg-Glu	NR
Asn-Arg-Glu	NR
Asp-Arg-Glu	NR
Cys-Arg-Glu	NR
Gln-Arg-Glu	NR
Glu-Arg-Glu	NR
Gly-Arg-Glu	NR
His-Arg-Glu	NR
Ile-Arg-Glu	NR
Leu-Arg-Glu	NR
Lys-Arg-Glu	NR
Met-Arg-Glu	NR



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Phe-Arg-Glu	NR
Pro-Arg-Glu	NR
Ser-Arg-Glu	NR
Thr-Arg-Glu	NR
Trp-Arg-Glu	NR
Tyr-Arg-Glu	NR
Val-Arg-Glu	NR
Glu-Arg-Ala	NR
Glu-Arg-Arg	NR
Glu-Arg-Asn	NR
Glu-Arg-Asp	NR
Glu-Arg-Cys	NR
Glu-Arg-Gln	NR
Glu-Arg-Gly	NR
Glu-Arg-His	NR
Glu-Arg-Ile	NR
Glu-Arg-Leu	NR
Glu-Arg-Lys	NR
Glu-Arg-Met	NR
Glu-Arg-Phe	NR
Glu-Arg-Pro	NR
Glu-Arg-Ser	NR
Glu-Arg-Thr	NR
Glu-Arg-Trp	NR
Glu-Arg-Tyr	NR
Glu-Arg-Val	NR

Table 20—Additional Exemplary Pharmacologically Active Peptides

Sequence/structure	SEQ ID NO:	Activity
VEPNCDIHVMWEWECFERL	1027	VEGF-antagonist
GERWCFDGLPTVWCGEES	1084	VEGF-antagonist
RGWVEICVADDNGMCVTEAQ	1085	VEGF-antagonist
GWDECDVARMWEWECFAGV	1086	VEGF- antagonist
GERWCFDGPRAWVCGWEI	501	VEGF- antagonist
EELWCFDGPRAWVCGYVK	502	VEGF- antagonist
RGWVEICAADDYGRCLTEAQ	1031	VEGF- antagonist
RGWVEICESDVWGRCL	1087	VEGF- antagonist
RGWVEICESDVWGRCL	1088	VEGF- antagonist
GGNECDIARMWEWECFERL	1089	VEGF- antagonist
RGWVEICAADDYGRCL	1090	VEGF-antagonist
CTTHWGF TLC	1028	MMP inhibitor
CLRSXGXC	1091	MMP inhibitor
CXXHWGFXXC	1092	MMP inhibitor
CXPXC	1093	MMP inhibitor
CRRHWGFEEC	1094	MMP inhibitor
STTHWGF TLS	1095	MMP inhibitor
CSLHWGFWWC	1096	CTLA4-mimetic
GFVCSGIFAVGVGRC	125	CTLA4-mimetic
APGVRLGCAVLGRYC	126	CTLA4-mimetic
LLGRMK	105	Antiviral (HBV)
ICVVQDWGHHRCTAGHMANLTSHASAI	127	C3b antagonist
ICVVQDWGHHRCT	128	C3b antagonist
CVVQDWGHHAC	129	C3b antagonist
STGGFDDVYDWARGVSSALTTTLVATR	185	Vinculin-binding
STGGFDDVYDWARRVSSALTTTLVATR	186	Vinculin-binding
SRGVNFSEWLYDMSAAMKEASNVFPSRRSR	187	Vinculin-binding
SSQNWDM EAGVEDLTAAMLGLLSTIHSSSR	188	Vinculin-binding
SSPSLYTOFLVNYESAATRIODLLIASRPSR	189	Vinculin-binding
SSTGWVDDLGLAQRAADATRTSIPPSLQNSR	190	Vinculin-binding
DVYTKKELIECARRVSEK	191	Vinculin-binding
EKGSYYPGSIAQFHIDYNNVS	192	C4BP-binding
SGIAQFHIDYNNVSSAEGWHVN	193	C4BP-binding
LVTVEKGSYYPGSGIAQFHIDYNNVSSAEGWHVN	194	C4BP-binding
SGIAQFHIDYNNVS	195	C4BP-binding
LLGRMK	279	anti-HBV
ALLGRMKMG	280	anti-HBV
LDPAFR	281	anti-HBV
CXXRGDC	322	Inhibition of platelet aggregation
RPLPLP	323	Src antagonist
PPVPPR	324	Src antagonist
XXFXDXWXXLXX	325	Anti-cancer (particularly for

		sarcomas)
KACRRFLGFPVDSEQLSRDCD	326	p16-mimetic
RERWNFDVFTETPLEGDFAW	327	p16-mimetic
KRRQTSMTDFYHSKRRLIFS	328	p16-mimetic
TSMTDFYHSKRRLIFSKRKP	329	p16-mimetic
RRLIF	330	p16-mimetic
KRRQTSATDFYHSKRRLIFSRQIKIWQNRRMKWKK	331	p16-mimetic
KRRLIFSKRQIKIWQNRRMKWKK	332	p16-mimetic
Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln	498	CAP37 mimetic/LPS binding
Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys	499	CAP37 mimetic/LPS binding
Gly Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val	500	CAP37 mimetic/LPS binding
WHWRHRIPLQLAAGR	1097	carbohydrate (GD1 alpha) mimetic
LKTPRV	1098	$\beta$ 2GPI Ab binding
NTLKTPRV	1099	$\beta$ 2GPI Ab binding
NTLKTPRVGGC	1100	$\beta$ 2GPI Ab binding
KDKATF	1101	$\beta$ 2GPI Ab binding
KDKATFGCHD	1102	$\beta$ 2GPI Ab binding
KDKATFGCHDGC	1103	$\beta$ 2GPI Ab binding
TLRVYK	1104	$\beta$ 2GPI Ab binding
ATLRVYKGG	1105	$\beta$ 2GPI Ab binding
CATLRVYKGG	1106	$\beta$ 2GPI Ab binding
INLKALAALAKKIL	1107	Membrane- transporting
GWT	NR	Membrane- transporting
GWTLNSAGYLLG	1108	Membrane- transporting
GWTLNSAGYLLGKINLKALAALAKKIL	1109	Membrane- transporting

The present invention is also particularly useful with peptides having activity in treatment of:

- cancer, wherein the peptide is a VEGF-mimetic or a VEGF receptor antagonist, a HER2 agonist or antagonist, a CD20 antagonist and the like;
- asthma, wherein the protein of interest is a CKR3 antagonist, an IL-5 receptor antagonist, and the like;
- thrombosis, wherein the protein of interest is a GPIIb antagonist, a GPIIIa antagonist, and the like;

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- autoimmune diseases and other conditions involving immune modulation, wherein the protein of interest is an IL-2 receptor antagonist, a CD40 agonist or antagonist, a CD40L agonist or antagonist, a thymopoietin mimetic and the like.

5        Vehicles. This invention requires the presence of at least one vehicle ( $F^1$ ,  $F^2$ ) attached to a peptide through the N-terminus, C-terminus or a sidechain of one of the amino acid residues. Multiple vehicles may also be used; e.g., Fc's at each terminus or an Fc at a terminus and a PEG group at the other terminus or a sidechain.

10        An Fc domain is the preferred vehicle. The Fc domain may be fused to the N or C termini of the peptides or at both the N and C termini. For the TPO-mimetic peptides, molecules having the Fc domain fused to the N terminus of the peptide portion of the molecule are more bioactive than other such fusions, so fusion to the N terminus is preferred.

15        As noted above, Fc variants are suitable vehicles within the scope of this invention. A native Fc may be extensively modified to form an Fc variant in accordance with this invention, provided binding to the salvage receptor is maintained; see, for example WO 97/34631 and WO 96/32478. In such Fc variants, one may remove one or more sites of a native Fc that  
20        provide structural features or functional activity not required by the fusion molecules of this invention. One may remove these sites by, for example, substituting or deleting residues, inserting residues into the site, or truncating portions containing the site. The inserted or substituted residues may also be altered amino acids, such as peptidomimetics or D-  
25        amino acids. Fc variants may be desirable for a number of reasons, several of which are described below. Exemplary Fc variants include molecules and sequences in which:

1. Sites involved in disulfide bond formation are removed. Such removal may avoid reaction with other cysteine-containing proteins present in

- the host cell used to produce the molecules of the invention. For this purpose, the cysteine-containing segment at the N-terminus may be truncated or cysteine residues may be deleted or substituted with other amino acids (e.g., alanyl, seryl). In particular, one may truncate the N-terminal 20-amino acid segment of SEQ ID NO: 2 or delete or substitute the cysteine residues at positions 7 and 10 of SEQ ID NO: 2. Even when cysteine residues are removed, the single chain Fc domains can still form a dimeric Fc domain that is held together non-covalently.
2. A native Fc is modified to make it more compatible with a selected host cell. For example, one may remove the PA sequence near the N-terminus of a typical native Fc, which may be recognized by a digestive enzyme in E. coli such as proline iminopeptidase. One may also add an N-terminal methionine residue, especially when the molecule is expressed recombinantly in a bacterial cell such as E. coli. The domain of SEQ ID NO: 2 (Figure 4) is one such Fc variant.
  3. A portion of the N-terminus of a native Fc is removed to prevent N-terminal heterogeneity when expressed in a selected host cell. For this purpose, one may delete any of the first 20 amino acid residues at the N-terminus, particularly those at positions 1, 2, 3, 4 and 5.
  4. One or more glycosylation sites are removed. Residues that are typically glycosylated (e.g., asparagine) may confer cytolytic response. Such residues may be deleted or substituted with unglycosylated residues (e.g., alanine).
  5. Sites involved in interaction with complement, such as the C1q binding site, are removed. For example, one may delete or substitute the EKK sequence of human IgG1. Complement recruitment may not be advantageous for the molecules of this invention and so may be avoided with such an Fc variant.

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6. Sites are removed that affect binding to Fc receptors other than a salvage receptor. A native Fc may have sites for interaction with certain white blood cells that are not required for the fusion molecules of the present invention and so may be removed.
- 5 7. The ADCC site is removed. ADCC sites are known in the art; see, for example, Molec. Immunol. 29 (5): 633-9 (1992) with regard to ADCC sites in IgG1. These sites, as well, are not required for the fusion molecules of the present invention and so may be removed.
8. When the native Fc is derived from a non-human antibody, the native  
10 Fc may be humanized. Typically, to humanize a native Fc, one will substitute selected residues in the non-human native Fc with residues that are normally found in human native Fc. Techniques for antibody humanization are well known in the art.

Preferred Fc variants include the following. In SEQ ID NO: 2  
15 (Figure 4) the leucine at position 15 may be substituted with glutamate; the glutamate at position 99, with alanine; and the lysines at positions 101 and 103, with alanines. In addition, one or more tyrosine residues can be replaced by phenylalanine residues.

An alternative vehicle would be a protein, polypeptide, peptide,  
20 antibody, antibody fragment, , or small molecule (e.g., a peptidomimetic compound) capable of binding to a salvage receptor. For example, one could use as a vehicle a polypeptide as described in U.S. Pat. No. 5,739,277, issued April 14, 1998 to Presta et al. Peptides could also be selected by  
25 phage display for binding to the FcRn salvage receptor. Such salvage receptor-binding compounds are also included within the meaning of "vehicle" and are within the scope of this invention. Such vehicles should be selected for increased half-life (e.g., by avoiding sequences recognized by proteases) and decreased immunogenicity (e.g., by favoring non-immunogenic sequences, as discovered in antibody humanization).

As noted above, polymer vehicles may also be used for F<sup>1</sup> and F<sup>2</sup>. Various means for attaching chemical moieties useful as vehicles are currently available, see, e.g., Patent Cooperation Treaty ("PCT") International Publication No. WO 96/11953, entitled "N-Terminally

5 Chemically Modified Protein Compositions and Methods," herein incorporated by reference in its entirety. This PCT publication discloses, among other things, the selective attachment of water soluble polymers to the N-terminus of proteins.

A preferred polymer vehicle is polyethylene glycol (PEG). The PEG

10 group may be of any convenient molecular weight and may be linear or branched. The average molecular weight of the PEG will preferably range from about 2 kiloDalton ("kD") to about 100 kDa, more preferably from about 5 kDa to about 50 kDa, most preferably from about 5 kDa to about 10 kDa. The PEG groups will generally be attached to the compounds of

15 the invention via acylation or reductive alkylation through a reactive group on the PEG moiety (e.g., an aldehyde, amino, thiol, or ester group) to a reactive group on the inventive compound (e.g., an aldehyde, amino, or ester group).

A useful strategy for the PEGylation of synthetic peptides consists

20 of combining, through forming a conjugate linkage in solution, a peptide and a PEG moiety, each bearing a special functionality that is mutually reactive toward the other. The peptides can be easily prepared with conventional solid phase synthesis (see, for example, Figures 5 and 6 and the accompanying text herein). The peptides are "preactivated" with an

25 appropriate functional group at a specific site. The precursors are purified and fully characterized prior to reacting with the PEG moiety. Ligation of the peptide with PEG usually takes place in aqueous phase and can be easily monitored by reverse phase analytical HPLC. The PEGylated peptides can be easily purified by preparative HPLC and characterized by

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analytical HPLC, amino acid analysis and laser desorption mass spectrometry.

Polysaccharide polymers are another type of water soluble polymer which may be used for protein modification. Dextran is polysaccharide polymers comprised of individual subunits of glucose predominantly linked by  $\alpha$ 1-6 linkages. The dextran itself is available in many molecular weight ranges, and is readily available in molecular weights from about 1 kD to about 70 kD. Dextran is a suitable water soluble polymer for use in the present invention as a vehicle by itself or in combination with another vehicle (e.g., Fc). See, for example, WO 96/11953 and WO 96/05309. The use of dextran conjugated to therapeutic or diagnostic immunoglobulins has been reported; see, for example, European Patent Publication No. 0 315 456, which is hereby incorporated by reference. Dextran of about 1 kD to about 20 kD is preferred when dextran is used as a vehicle in accordance with the present invention.

Linkers. Any "linker" group is optional. When present, its chemical structure is not critical, since it serves primarily as a spacer. The linker is preferably made up of amino acids linked together by peptide bonds. Thus, in preferred embodiments, the linker is made up of from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids. Some of these amino acids may be glycosylated, as is well understood by those in the art. In a more preferred embodiment, the 1 to 20 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine. Even more preferably, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. Thus, preferred linkers are polyglycines (particularly (Gly)<sub>n</sub>, (Gly)<sub>3</sub>), poly(Gly-Ala), and polyalanines. Other specific examples of linkers are:

(Gly)<sub>3</sub>Lys(Gly), (SEQ ID NO: 333);



(Gly)<sub>3</sub>AsnGlySer(Gly)<sub>2</sub> (SEQ ID NO: 334);

(Gly)<sub>3</sub>Cys(Gly)<sub>4</sub> (SEQ ID NO: 335); and

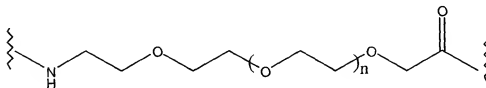
GlyProAsnGlyGly (SEQ ID NO: 336).

To explain the above nomenclature, for example, (Gly)<sub>3</sub>Lys(Gly)<sub>1</sub> means

- 5 Gly-Gly-Gly-Lys-Gly-Gly-Gly. Combinations of Gly and Ala are also preferred. The linkers shown here are exemplary; linkers within the scope of this invention may be much longer and may include other residues.

Non-peptide linkers are also possible. For example, alkyl linkers such as -NH-(CH<sub>2</sub>)<sub>s</sub>-C(O)-, wherein s = 2-20 could be used. These alkyl

- 10 linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C<sub>1</sub>-C<sub>6</sub>), lower acyl, halogen (e.g., Cl, Br), CN, NH<sub>2</sub>, phenyl, etc. An exemplary non-peptide linker is a PEG linker,  
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15

wherein n is such that the linker has a molecular weight of 100 to 5000 kD, preferably 100 to 500 kD. The peptide linkers may be altered to form derivatives in the same manner as described above.

Derivatives. The inventors also contemplate derivatizing the

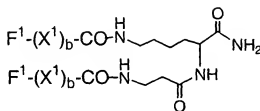
- 20 peptide and/or vehicle portion of the compounds. Such derivatives may improve the solubility, absorption, biological half life, and the like of the compounds. The moieties may alternatively eliminate or attenuate any undesirable side-effect of the compounds and the like. Exemplary derivatives include compounds in which:

- 25 1. The compound or some portion thereof is cyclic. For example, the peptide portion may be modified to contain two or more Cys residues (e.g., in the linker), which could cyclize by disulfide bond formation.

For citations to references on preparation of cyclized derivatives, see Table 2.

2. The compound is cross-linked or is rendered capable of cross-linking between molecules. For example, the peptide portion may be modified to contain one Cys residue and thereby be able to form an intermolecular disulfide bond with a like molecule. The compound may also be cross-linked through its C-terminus, as in the molecule shown below.

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3. One or more peptidyl [-C(O)NR-] linkages (bonds) is replaced by a non-peptidyl linkage. Exemplary non-peptidyl linkages are -CH<sub>2</sub>-carbamate [-CH<sub>2</sub>-OC(O)NR-], phosphonate, -CH<sub>2</sub>-sulfonamide [-CH<sub>2</sub>-S(O)<sub>2</sub>NR-], urea [-NHC(O)NH-], -CH<sub>2</sub>-secondary amine, and alkylated peptide [-C(O)NR<sup>6</sup>- wherein R<sup>6</sup> is lower alkyl].
5. The N-terminus is derivatized. Typically, the N-terminus may be acylated or modified to a substituted amine. Exemplary N-terminal derivative groups include -NRR<sup>1</sup> (other than -NH<sub>2</sub>), -NRC(O)R<sup>1</sup>, -NRC(O)OR<sup>1</sup>, -NRS(O)<sub>2</sub>R<sup>1</sup>, -NHC(O)NHR<sup>1</sup>, succinimide, or benzyloxycarbonyl-NH- (CBZ-NH-), wherein R and R<sup>1</sup> are each independently hydrogen or lower alkyl and wherein the phenyl ring may be substituted with 1 to 3 substituents selected from the group consisting of C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>1</sub>-C<sub>4</sub> alkoxy, chloro, and bromo.
6. The free C-terminus is derivatized. Typically, the C-terminus is esterified or amidated. For example, one may use methods described in the art to add (NH-CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>)<sub>n</sub> to compounds of this invention

- having any of SEQ ID NOS: 504 to 508 at the C-terminus. Likewise, one may use methods described in the art to add -NH<sub>2</sub> to compounds of this invention having any of SEQ ID NOS: 924 to 955, 963 to 972, 1005 to 1013, or 1018 to 1023 at the C-terminus. Exemplary C-terminal derivative groups include, for example, -C(O)R<sup>2</sup> wherein R<sup>2</sup> is lower alkoxy or -NR<sup>3</sup>R<sup>4</sup> wherein R<sup>3</sup> and R<sup>4</sup> are independently hydrogen or C<sub>1</sub>-C<sub>8</sub> alkyl (preferably C<sub>1</sub>-C<sub>4</sub> alkyl).
7. A disulfide bond is replaced with another, preferably more stable, cross-linking moiety (e.g., an alkylene). See, e.g., Bhatnagar et al. (1996), J. Med. Chem. 39: 3814-9; Alberts et al. (1993) Thirteenth Am. Pep. Symp., 357-9.
8. One or more individual amino acid residues is modified. Various derivatizing agents are known to react specifically with selected sidechains or terminal residues, as described in detail below.
- 15 Lysinyl residues and amino terminal residues may be reacted with succinic or other carboxylic acid anhydrides, which reverse the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.
- 20 Arginyl residues may be modified by reaction with any one or combination of several conventional reagents, including phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginyl residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.
- 25

Specific modification of tyrosyl residues has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl  
5 tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl sidechain groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides ( $R'-N=C=N-R'$ ) such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues  
10 may be converted to asparaginy and glutaminy residues by reaction with ammonium ions.

Glutaminy and asparaginy residues may be deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues  
15 falls within the scope of this invention.

Cysteinyl residues can be replaced by amino acid residues or other moieties either to eliminate disulfide bonding or, conversely, to stabilize cross-linking. See, e.g., Bhatnagar *et al.* (1996), *J. Med. Chem.* 39: 3814-9.

Derivatization with bifunctional agents is useful for cross-linking the  
20 peptides or their functional derivatives to a water-insoluble support matrix or to other macromolecular vehicles. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-  
25 dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates

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and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Carbohydrate (oligosaccharide) groups may conveniently be attached to sites that are known to be glycosylation sites in proteins. Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids other than proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (e.g., in mammalian cells such as CHO, BHK, COS). However, such sites may further be glycosylated by synthetic or semi-synthetic procedures known in the art.

Other possible modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, oxidation of the sulfur atom in Cys, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains. Creighton, Proteins: Structure and Molecule Properties (W. H. Freeman & Co., San Francisco), pp. 79-86 (1983).

Compounds of the present invention may be changed at the DNA level, as well. The DNA sequence of any portion of the compound may be

changed to codons more compatible with the chosen host cell. For E. coli, which is the preferred host cell, optimized codons are known in the art. Codons may be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected  
5 host cell. The vehicle, linker and peptide DNA sequences may be modified to include any of the foregoing sequence changes.

#### **Methods of Making**

The compounds of this invention largely may be made in transformed host cells using recombinant DNA techniques. To do so, a  
10 recombinant DNA molecule coding for the peptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from DNA using suitable restriction enzymes. Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the  
15 phosphoramidate method. Also, a combination of these techniques could be used.

The invention also includes a vector capable of expressing the peptides in an appropriate host. The vector comprises the DNA molecule that codes for the peptides operatively linked to appropriate expression  
20 control sequences. Methods of effecting this operative linking, either before or after the DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the  
25 control of transcription or translation.

The resulting vector having the DNA molecule thereon is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial hosts include bacteria (such as E. coli sp.), yeast (such as Saccharomyces sp.) and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

Next, the transformed host is cultured and purified. Host cells may be cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art. Finally, the peptides are purified from culture by methods well known in the art.

The compounds may also be made by synthetic methods. For example, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield (1973), Chem. Polypeptides, pp. 335-61 (Katsoyannis and Panayotis eds.); Merrifield (1963), J. Am. Chem. Soc. 85: 2149; Davis et al. (1985), Biochem. Intl. 10: 394-414; Stewart and Young (1969), Solid Phase Peptide Synthesis; U.S. Pat. No. 3,941,763; Finn et al. (1976), The Proteins (3rd ed.) 2: 105-253; and Erickson et al. (1976), The Proteins (3rd ed.) 2: 257-527. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

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Compounds that contain derivatized peptides or which contain non-peptide groups may be synthesized by well-known organic chemistry techniques.



### Uses of the Compounds

In general. The compounds of this invention have pharmacologic activity resulting from their ability to bind to proteins of interest as agonists, mimetics or antagonists of the native ligands of such proteins of interest. The utility of specific compounds is shown in Table 2. The activity of these compounds can be measured by assays known in the art. For the TPO-mimetic and EPO-mimetic compounds, in vivo assays are further described in the Examples section herein.

In addition to therapeutic uses, the compounds of the present invention are useful in diagnosing diseases characterized by dysfunction of their associated protein of interest. In one embodiment, a method of detecting in a biological sample a protein of interest (e.g., a receptor) that is capable of being activated comprising the steps of: (a) contacting the sample with a compound of this invention; and (b) detecting activation of the protein of interest by the compound. The biological samples include tissue specimens, intact cells, or extracts thereof. The compounds of this invention may be used as part of a diagnostic kit to detect the presence of their associated proteins of interest in a biological sample. Such kits employ the compounds of the invention having an attached label to allow for detection. The compounds are useful for identifying normal or abnormal proteins of interest. For the EPO-mimetic compounds, for example, presence of abnormal protein of interest in a biological sample may be indicative of such disorders as Diamond Blackfan anemia, where it is believed that the EPO receptor is dysfunctional.

Therapeutic uses of EPO-mimetic compounds. The EPO-mimetic compounds of the invention are useful for treating disorders characterized by low red blood cell levels. Included in the invention are methods of modulating the endogenous activity of an EPO receptor in a mammal, preferably methods of increasing the activity of an EPO receptor. In

general, any condition treatable by erythropoietin, such as anemia, may also be treated by the EPO-mimetic compounds of the invention. These compounds are administered by an amount and route of delivery that is appropriate for the nature and severity of the condition being treated and  
5 may be ascertained by one skilled in the art. Preferably, administration is by injection, either subcutaneous, intramuscular, or intravenous.

Therapeutic uses of TPO-mimetic compounds. For the TPO-mimetic compounds, one can utilize such standard assays as those described in WO95/26746 entitled "Compositions and Methods for  
10 Stimulating Megakaryocyte Growth and Differentiation". In vivo assays also appear in the Examples hereinafter.

The conditions to be treated are generally those that involve an existing megakaryocyte/platelet deficiency or an expected megakaryocyte/platelet deficiency (e.g., because of planned surgery or  
15 platelet donation). Such conditions will usually be the result of a deficiency (temporary or permanent) of active Mpl ligand in vivo. The generic term for platelet deficiency is thrombocytopenia, and hence the methods and compositions of the present invention are generally available for treating thrombocytopenia in patients in need thereof.

Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other  
20 specific disease conditions. Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia, idiopathic thrombocytopenia, metastatic  
25 tumors which result in thrombocytopenia, systemic lupus erythematosus, splenomegaly, Fanconi's syndrome, vitamin B12 deficiency, folic acid deficiency, May-Hegglin anomaly, Wiskott-Aldrich syndrome, and paroxysmal nocturnal hemoglobinuria. Also, certain treatments for AIDS

result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet deficiencies, e.g., due to future surgery, a compound of the present invention could be administered  
5 several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, a compound of this invention could be administered along with blood or purified platelets.

The TPO-mimetic compounds of this invention may also be useful in  
10 stimulating certain cell types other than megakaryocytes if such cells are found to express Mpl receptor. Conditions associated with such cells that express the Mpl receptor, which are responsive to stimulation by the Mpl ligand, are also within the scope of this invention.

The TPO-mimetic compounds of this invention may be used in any  
15 situation in which production of platelets or platelet precursor cells is desired, or in which stimulation of the c-Mpl receptor is desired. Thus, for example, the compounds of this invention may be used to treat any condition in a mammal wherein there is a need of platelets, megakaryocytes, and the like. Such conditions are described in detail in the following exemplary sources:  
20 WO95/26746; WO95/21919; WO95/18858; WO95/21920 and are incorporated herein.

The TPO-mimetic compounds of this invention may also be useful in maintaining the viability or storage life of platelets and/or megakaryocytes and related cells. Accordingly, it could be useful to include an effective amount of  
25 one or more such compounds in a composition containing such cells.

The therapeutic methods, compositions and compounds of the present invention may also be employed, alone or in combination with other cytokines, soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies in the treatment of disease states

- characterized by other symptoms as well as platelet deficiencies. It is anticipated that the inventive compound will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic
- 5 stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed with Mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6,
- 10 IL-11, colony stimulating factor-1 (CSF-1), SCF, GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), consensus interferon, IFN-beta, or IFN-gamma. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of
- 15 causing megakaryocytes to fragment into platelets once the megakaryocytes have reached mature form. Thus, administration of an inventive compound (to enhance the number of mature megakaryocytes) followed by administration of the soluble Mpl receptor (to inactivate the ligand and allow the mature megakaryocytes to produce platelets) is
- 20 expected to be a particularly effective means of stimulating platelet production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

- In cases where the inventive compounds are added to compositions
- 25 of platelets and/or megakaryocytes and related cells, the amount to be included will generally be ascertained experimentally by techniques and assays known in the art. An exemplary range of amounts is 0.1  $\mu\text{g}$ —1 mg inventive compound per  $10^6$  cells.

### **Pharmaceutical Compositions**

- In General. The present invention also provides methods of using pharmaceutical compositions of the inventive compounds. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal, transdermal or other forms of administration. In general, the invention encompasses pharmaceutical compositions comprising effective amounts of a compound of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. See, *e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.
- Oral dosage forms. Contemplated for use herein are oral solid dosage forms, which are described generally in Chapter 89 of Remington's Pharmaceutical Sciences (1990), 18th Ed., Mack Publishing Co. Easton PA 18042, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also,

liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Patent 5 No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given in Chapter 10 of Marshall, K., Modern Pharmaceutics (1979), edited by G. S. Banker and C. T. Rhodes, herein incorporated by reference. In general, the formulation will include the inventive compound, and inert ingredients which allow for protection against the 10 stomach environment, and release of the biologically active material in the intestine.

Also specifically contemplated are oral dosage forms of the above inventive compounds. If necessary, the compounds may be chemically modified so that oral delivery is efficacious. Generally, the chemical 15 modification contemplated is the attachment of at least one moiety to the compound molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the compound and increase in circulation time in the body. Moieties useful as covalently 20 attached vehicles in this invention may also be used for this purpose. Examples of such moieties include: PEG, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline. See, for example, Abuchowski and Davis, Soluble Polymer-Enzyme Adducts, Enzymes as Drugs (1981), 25 Hoenberg and Roberts, eds., Wiley-Interscience, New York, NY, , pp 367-83; Newmark, et al. (1982), J. Appl. Biochem. 4:185-9. Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are PEG moieties.

For oral delivery dosage forms, it is also possible to use a salt of a modified aliphatic amino acid, such as sodium N-(8-[2-hydroxybenzoyl] amino) caprylate (SNAC), as a carrier to enhance absorption of the therapeutic compounds of this invention. The clinical efficacy of a heparin  
5 formulation using SNAC has been demonstrated in a Phase II trial conducted by Emisphere Technologies. See US Patent No. 5,792,451, "Oral drug delivery composition and methods".

The compounds of this invention can be included in the formulation as fine multiparticulates in the form of granules or pellets of  
10 particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or  
15 microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the compound of the invention with an inert material. These diluents could include  
20 carbohydrates, especially mannitol,  $\alpha$ -lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

25 Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange

peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the compound of this invention into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or



benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives may also be included in the formulation to enhance uptake of the compound. Additives potentially having this property are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release formulation may be desirable. The compound of this invention could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms e.g., gums. Slowly degenerating matrices may also be incorporated into the formulation, e.g., alginates, polysaccharides. Another form of a controlled release of the compounds of this invention is by a method based on the Oros therapeutic system (Alza Corp.), i.e., the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

- Pulmonary delivery forms. Also contemplated herein is pulmonary
- 5 delivery of the present protein (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei *et al.*, Pharma. Res. (1990) 7: 565-9; Adjei *et al.* (1990), Internatl. J. Pharmaceutics 63: 135-44 (leuprolide acetate); Braquet
- 10 *et al.* (1989), J. Cardiovasc. Pharmacol. 13 (suppl.5): s.143-146 (endothelin-1); Hubbard *et al.* (1989), Annals Int. Med. 3: 206-12 ( $\alpha$ 1-antitrypsin); Smith *et al.* (1989), J. Clin. Invest. 84: 1145-6 ( $\alpha$ 1-proteinase); Oswein *et al.* (March 1990), "Aerosolization of Proteins", Proc. Symp. Resp. Drug Delivery II, Keystone, Colorado (recombinant human growth hormone); Debs *et al.*
- 15 (1988), J. Immunol. 140: 3482-8 (interferon- $\gamma$  and tumor necrosis factor  $\alpha$ ) and Platz *et al.*, U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor).

- Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of
- 20 therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the
- 25 Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of the inventive compound. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to diluents, adjuvants  
5 and/or carriers useful in therapy.

The inventive compound should most advantageously be prepared in particulate form with an average particle size of less than 10  $\mu\text{m}$  (or microns), most preferably 0.5 to 5  $\mu\text{m}$ , for most effective delivery to the distal lung.

10 Pharmaceutically acceptable carriers include carbohydrates such as trehalose, mannitol, xylitol, sucrose, lactose, and sorbitol. Other ingredients for use in formulations may include DPPC, DOPE, DSPC and DOPC. Natural or synthetic surfactants may be used. PEG may be used (even apart from its use in derivatizing the protein or analog). Dextran,  
15 such as cyclodextran, may be used. Bile salts and other related enhancers may be used. Cellulose and cellulose derivatives may be used. Amino acids may be used, such as use in a buffer formulation.

Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated.

20 Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the inventive compound dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic  
25 pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the inventive

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compound suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing the inventive compound and may also include a bulking agent, such as lactose, sorbitol, sucrose, mannitol, trehalose, or xylitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation.

Nasal delivery forms. Nasal delivery of the inventive compound is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran. Delivery via transport across other mucous membranes is also contemplated.

Dosages. The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 0.1-1000 micrograms of the inventive compound per kilogram of body weight, preferably 0.1-150 micrograms per kilogram.

### Specific preferred embodiments

- The inventors have determined preferred peptide sequences for molecules having many different kinds of activity. The inventors have further determined preferred structures of these preferred peptides
- 5 combined with preferred linkers and vehicles. Preferred structures for these preferred peptides listed in Table 21 below.

**Table 21—Preferred embodiments**

Sequence/structure	SEQ ID NO:	Activity
F <sup>1</sup> -(G) <sub>5</sub> -IEGPTLRQWLAARA-(G) <sub>5</sub> -IEGPTLRQWLAARA	337	TPO-mimetic
IEGPTLRQWLAARA-(G) <sub>5</sub> -IEGPTLRQWLAARA-(G) <sub>5</sub> -F <sup>1</sup>	338	TPO-mimetic
F <sup>1</sup> -(G) <sub>5</sub> -IEGPTLRQWLAARA	1032	TPO-mimetic
IEGPTLRQWLAARA -(G) <sub>5</sub> -F <sup>1</sup>	1033	TPO-mimetic
F <sup>1</sup> -(G) <sub>5</sub> -GGTYSCHFGPLTWVCKPOGG-(G) <sub>4</sub> -GGTYSCHFGPLTWVCKPOGG	339	EPO-mimetic
GGTYSCHFGPLTWVCKPOGG-(G) <sub>4</sub> -GGTYSCHFGPLTWVCKPOGG-(G) <sub>5</sub> -F <sup>1</sup>	340	EPO-mimetic
GGTYSCHFGPLTWVCKPOGG-(G) <sub>5</sub> -F <sup>1</sup>	1034	EPO-mimetic
F <sup>1</sup> -(G) <sub>5</sub> -DFLPHYKNTSLGHRP	1045	TNF-α inhibitor
DFLPHYKNTSLGHRP-(G) <sub>5</sub> -F <sup>1</sup>	1046	TNF-α inhibitor
F <sup>1</sup> -(G) <sub>5</sub> -FEWTPGYWQPYALPL	1047	IL-1 R antagonist
FEWTPGYWQPYALPL-(G) <sub>5</sub> -F <sup>1</sup>	1048	IL-1 R antagonist
F <sup>1</sup> -(G) <sub>5</sub> -VEPNCDIHVMWEWECFERL	1049	VEGF-antagonist
VEPNCDIHVMWEWECFERL-(G) <sub>5</sub> -F <sup>1</sup>	1050	VEGF-antagonist
F <sup>1</sup> -(G) <sub>5</sub> -CTTHWGFTLC	1051	MMP inhibitor
CTTHWGFTLC-(G) <sub>5</sub> -F <sup>1</sup>	1052	MMP inhibitor

"F<sup>1</sup>" is an Fc domain as defined previously herein.

### Working examples

The compounds described above may be prepared as described below. These examples comprise preferred embodiments of the invention and are illustrative rather than limiting.

**Example 1**

**TPO-Mimetics**

The following example uses peptides identified by the numbers appearing in Table A hereinafter.

Preparation of peptide 19. Peptide 17b (12 mg) and MeO-PEG-SH 5000 (30 mg, 2 equiv.) were dissolved in 1 ml aqueous buffer (pH 8). The mixture was incubated at RT for about 30 minutes and the reaction was checked by analytical HPLC, which showed a > 80% completion of the reaction. The pegylated material was isolated by preparative HPLC.

Preparation of peptide 20. Peptide 18 (14 mg) and MeO-PEG-maleimide (25 mg) were dissolved in about 1.5 ml aqueous buffer (pH 8). The mixture was incubated at RT for about 30 minutes, at which time about 70% transformation was complete as monitored with analytical HPLC by applying an aliquot of sample to the HPLC column. The pegylated material was purified by preparative HPLC.

Bioactivity assay. The TPO in vitro bioassay is a mitogenic assay utilizing an IL-3 dependent clone of murine 32D cells that have been transfected with human mpl receptor. This assay is described in greater detail in WO 95/26746. Cells are maintained in MEM medium containing 10% Fetal Clone II and 1 ng/ml mIL-3. Prior to sample addition, cells are prepared by rinsing twice with growth medium lacking mIL-3. An extended twelve point TPO standard curve is prepared, ranging from 33 to 39 pg/ml. Four dilutions, estimated to fall within the linear portion of the standard curve, (100 to 125 pg/ml), are prepared for each sample and run in triplicate. A volume of 100  $\mu$ l of each dilution of sample or standard is added to appropriate wells of a 96 well microtiter plate

containing 10,000 cells/well. After forty-four hours at 37 °C and 10% CO<sub>2</sub>, MTS (a tetrazolium compound which is bio-reduced by cells to a formazan) is added to each well. Approximately six hours later, the optical density is read on a plate reader at 490 nm. A dose response curve (log TPO

- 5 concentration vs. O.D.- Background) is generated and linear regression analysis of points which fall in the linear portion of the standard curve is performed. Concentrations of unknown test samples are determined using the resulting linear equation and a correction for the dilution factor.

- TMP tandem repeats with polyglycine linkers. Our design of
- 10 sequentially linked TMP repeats was based on the assumption that a dimeric form of TMP was required for its effective interaction with c-Mpl (the TPO receptor) and that depending on how they were wound up against each other in the receptor context, the two TMP molecules could be tethered together in the C- to N-terminus configuration in a way that
- 15 would not perturb the global dimeric conformation. Clearly, the success of the design of tandem linked repeats depends on proper selection of the length and composition of the linker that joins the C- and N-termini of the two sequentially aligned TMP monomers. Since no structural information of the TMP bound to c-Mpl was available, a series of repeated peptides
- 20 with linkers composed of 0 to 10 and 14 glycine residues (Table A) were synthesized. Glycine was chosen because of its simplicity and flexibility, based on the rationale that a flexible polyglycine peptide chain might allow for the free folding of the two tethered TMP repeats into the required conformation, while other amino acid sequences may adopt
- 25 undesired secondary structures whose rigidity might disrupt the correct packing of the repeated peptide in the receptor context.

The resulting peptides are readily accessible by conventional solid phase peptide synthesis methods (Merrifield (1963), J. Amer. Chem. Soc. 85: 2149) with either Fmoc or t-Boc chemistry. Unlike the synthesis of the

C-terminally linked parallel dimer which required the use of an orthogonally protected lysine residue as the initial branch point to build the two peptide chains in a pseudosymmetrical way (Cwirla et al. (1997), Science 276: 1696-9), the synthesis of these tandem repeats was a straightforward, stepwise assembly of the continuous peptide chains from the C- to N-terminus. Since dimerization of TMP had a more dramatic effect on the proliferative activity than binding affinity as shown for the C-terminal dimer (Cwirla et al. (1997)), the synthetic peptides were tested directly for biological activity in a TPO-dependent cell-proliferation assay using an IL-3 dependent clone of murine 32D cells transfected with the full-length c-Mpl (Palacios et al., Cell 41:727 (1985)). As the test results showed, all the polyglycine linked tandem repeats demonstrated >1000 fold increases in potency as compared to the monomer, and were even more potent than the C-terminal dimer in this cell proliferation assay. The absolute activity of the C-terminal dimer in our assay was lower than that of the native TPO protein, which is different from the previously reported findings in which the C-terminal dimer was found to be as active as the natural ligand (Cwirla et al. (1997)). This might be due to differences in the conditions used in the two assays. Nevertheless, the difference in activity between tandem (C terminal of first monomer linked to N terminal of second monomer) and C-terminal (C terminal of first monomer linked to C terminal of second monomer; also referred to as parallel) dimers in the same assay clearly demonstrated the superiority of tandem repeat strategy over parallel peptide dimerization. It is interesting to note that a wide range of length is tolerated by the linker. The optimal linker between tandem peptides with the selected TMP monomers apparently is composed of 8 glycines.

Other tandem repeats. Subsequent to this first series of TMP tandem repeats, several other molecules were designed either with



different linkers or containing modifications within the monomer itself. The first of these molecules, peptide 13, has a linker composed of GPNG, a sequence known to have a high propensity to form a  $\beta$ -turn-type secondary structure. Although still about 100-fold more potent than the monomer, this peptide was found to be >10-fold less active than the equivalent GGGG-linked analog. Thus, introduction of a relatively rigid  $\beta$ -turn at the linker region seemed to have caused a slight distortion of the optimal agonist conformation in this short linker form.

The Trp9 in the TMP sequence is a highly conserved residue among the active peptides isolated from random peptide libraries. There is also a highly conserved Trp in the consensus sequences of EPO mimetic peptides and this Trp residue was found to be involved in the formation of a hydrophobic core between the two EMPs and contributed to hydrophobic interactions with the EPO receptor. Livnah *et al.* (1996), *Science* 273: 464-71). By analogy, the Trp9 residue in TMP might have a similar function in dimerization of the peptide ligand, and as an attempt to modulate and estimate the effects of noncovalent hydrophobic forces exerted by the two indole rings, several analogs were made resulting from mutations at the Trp. So in peptide 14, the Trp residue was replaced in each of the two TMP monomers with a Cys, and an intramolecular disulfide bond was formed between the two cysteines by oxidation which was envisioned to mimic the hydrophobic interactions between the two Trp residues in peptide dimerization. Peptide 15 is the reduced form of peptide 14. In peptide 16, the two Trp residues were replaced by Ala. As the assay data show, all three analogs were inactive. These data further demonstrated that Trp is critical for the activity of the TPO mimetic peptide, not just for dimer formation.

The next two peptides (peptide 17a, and 18) each contain in their 8-amino acid linker a Lys or Cys residue. These two compounds are

precursors to the two PEGylated peptides (peptide 19 and 20) in which the side chain of the Lys or Cys is modified by a PEG moiety. A PEG moiety was introduced at the middle of a relatively long linker, so that the large PEG component (5 kDa) is far enough away from the critical binding sites  
5 in the peptide molecule. PEG is a known biocompatible polymer which is increasingly used as a covalent modifier to improve the pharmacokinetic profiles of peptide- and protein-based therapeutics.

A modular, solution-based method was devised for convenient PEGylation of synthetic or recombinant peptides. The method is based on  
10 the now well established chemoselective ligation strategy which utilizes the specific reaction between a pair of mutually reactive functionalities. So, for pegylated peptide 19, the lysine side chain was preactivated with a bromoacetyl group to give peptide 17b to accommodate reaction with a thiol-derivatized PEG. To do that, an orthogonal protecting group, Dde,  
15 was employed for the protection of the lysine  $\epsilon$ -amine. Once the whole peptide chain was assembled, the N-terminal amine was reprotected with t-Boc. Dde was then removed to allow for the bromoacetylation. This strategy gave a high quality crude peptide which was easily purified using conventional reverse phase HPLC. Ligation of the peptide with the thiol-  
20 modified PEG took place in aqueous buffer at pH 8 and the reaction completed within 30 minutes. MALDI-MS analysis of the purified, pegylated material revealed a characteristic, bell-shaped spectrum with an increment of 44 Da between the adjacent peaks. For PEG-peptide 20, a cysteine residue was placed in the linker region and its side chain thiol  
25 group would serve as an attachment site for a maleimide-containing PEG. Similar conditions were used for the pegylation of this peptide. As the assay data revealed, these two pegylated peptides had even higher in vitro bioactivity as compared to their unpegylated counterparts.

Peptide 21 has in its 8-amino acid linker a potential glycosylation motif, NGS. Since our exemplary tandem repeats are made up of natural amino acids linked by peptide bonds, expression of such a molecule in an appropriate eukaryotic cell system should produce a glycopeptide with the carbohydrate moiety added on the side chain carboxamide of Asn. Glycosylation is a common post-translational modification process which can have many positive impacts on the biological activity of a given protein by increasing its aqueous solubility and *in vivo* stability. As the assay data show, incorporation of this glycosylation motif into the linker maintained high bioactivity. The synthetic precursor of the potential glycopeptide had in effect an activity comparable to that of the -(G)<sub>8</sub>-linked analog. Once glycosylated, this peptide is expected to have the same order of activity as the pegylated peptides, because of the similar chemophysical properties exhibited by a PEG and a carbohydrate moiety.

The last peptide is a dimer of a tandem repeat. It was prepared by oxidizing peptide 18, which formed an intermolecular disulfide bond between the two cysteine residues located at the linker. This peptide was designed to address the possibility that TMP was active as a tetramer. The assay data showed that this peptide was not more active than an average tandem repeat on an adjusted molar basis, which indirectly supports the idea that the active form of TMP is indeed a dimer, otherwise dimerization of a tandem repeat would have a further impact on the bioactivity.

In order to confirm the *in vitro* data in animals, one pegylated TMP tandem repeat (compound 20 in Table A) was delivered subcutaneously to normal mice via osmotic pumps. Time and dose-dependent increases were seen in platelet numbers for the duration of treatment. Peak platelet levels over 4-fold baseline were seen on day 8. A dose of 10 µg/kg/day of the pegylated TMP repeat produced a similar response to rHuMGDF (non-pegylated) at 100 µg/kg/day delivered by the same route.

Table A—TPO-mimetic Peptides

Peptide No.	Compound	SEQ ID NO:	Relative Potency
	TPO		++++
	TMP monomer	13	+
	TMP C-C dimer		+++
TMP-(G) <sub>n</sub> -TMP:			
1	n = 0	341	++++
2	n = 1	342	++++
3	n = 2	343	++++
4	n = 3	344	++++
5	n = 4	345	++++
6	n = 5	346	++++
7	n = 6	347	++++
8	n = 7	348	++++
9	n = 8	349	++++
10	n = 9	350	++++
11	n = 10	351	++++
12	n = 14	352	++++
13	TMP-GPNG-TMP	353	+++
14	IEGPTLRQCLAARA-GGGGGGGG-IEGPTLRQCLAARA (cyclic)	354	-
15	IEGPTLRQCLAARA-GGGGGGGG- IEGPTLRQCLAARA (linear)	355	-
16	IEGPTLRQALAARA-GGGGGGGG- IEGPTLRQALAARA	356	-
17a	TMP-GGGKGGGG-TMP	357	++++
17b	TMP-GGGK(BrAc)GGGG-TMP	358	ND
18	TMP-GGGCGGGG-TMP	359	++++
19	TMP-GGGK(PEG)GGGG-TMP	360	++++
20	TMP-GGGC(PEG)GGGG-TMP	361	++++
21	TMP-GGGN*GSGG-TMP	362	++++
22	TMP-GGGCGGGG-TMP	363	++++
	TMP-GGGCGGGG-TMP	363	++++

- Discussion. It is well accepted that MGDF acts in a way similar to hGH, i.e., one molecule of the protein ligand binds two molecules of the receptor for its activation. Wells et al. (1996), Ann. Rev. Biochem. 65: 609-34. Now, this interaction is mimicked by the action of a much smaller peptide, TMP. However, the present studies suggest that this mimicry requires the concerted action of two TMP molecules, as covalent dimerization of TMP in either a C-C parallel or C-N sequential fashion increased the in vitro biological potency of the original monomer by a factor of greater than  $10^3$ . The relatively low biopotency of the monomer is probably due to inefficient formation of the noncovalent dimer. A preformed covalent repeat has the ability to eliminate the entropy barrier for the formation of a noncovalent dimer which is exclusively driven by weak, noncovalent interactions between two molecules of the small, 14-residue peptide.
- It is intriguing that this tandem repeat approach had a similar effect on enhancing bioactivity as the reported C-C dimerization is intriguing. These two strategies brought about two very different molecular configurations. The C-C dimer is a quasi-symmetrical molecule, while the tandem repeats have no such symmetry in their linear structures. Despite this difference in their primary structures, these two types of molecules appeared able to fold effectively into a similar biologically active conformation and cause the dimerization and activation of c-Mpl. These experimental observations provide a number of insights into how the two TMP molecules may interact with one another in binding to c-Mpl. First, the two C-termini of the two bound TMP molecules must be in relatively close proximity with each other, as suggested by data on the C-terminal dimer. Second, the respective N- and C-termini of the two TMP molecules in the receptor complex must also be very closely aligned with each other, such that they can be directly tethered together with a single peptide bond

to realize the near maximum activity-enhancing effect brought about by the tandem repeat strategy. Insertion of one or more (up to 14) glycine residues at the junction did not increase (or decrease) significantly the activity any further. This may be due to the fact that a flexible polyglycine peptide chain is able to loop out easily from the junction without causing any significant changes in the overall conformation. This flexibility seems to provide the freedom of orientation for the TMP peptide chains to fold into the required conformation in interacting with the receptor and validate it as a site of modification. Indirect evidence supporting this came from the study on peptide 13, in which a much more rigid b-turn-forming sequence as the linker apparently forced a deviation of the backbone alignment around the linker which might have resulted in a slight distortion of the optimal conformation, thus resulting in a moderate (10-fold) decrease in activity as compared with the analogous compound with a 4-Gly linker. Third, Trp9 in TMP plays a similar role as Trp13 in EMP, which is involved not only in peptide:peptide interaction for the formation of dimers but also is important for contributing hydrophobic forces in peptide:receptor interaction. Results obtained with the W to C mutant analog, peptide 14, suggest that a covalent disulfide linkage is not sufficient to approximate the hydrophobic interactions provided by the Trp pair and that, being a short linkage, it might bring the two TMP monomers too close, therefore perturbing the overall conformation of the optimal dimeric structure.

An analysis of the possible secondary structure of the TMP peptide can provide further understanding on the interaction between TMP and c-Mpl. This can be facilitated by making reference to the reported structure of the EPO mimetic peptide. Livnah *et al.* (1996), *Science* 273:464-75 The receptor-bound EMP has a b-hairpin structure with a b-turn formed by the highly consensus Gly-Pro-Leu-Thr at the center of its sequence. Instead of

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GPLT, TMP has a highly selected GPTL sequence which is likely to form a similar turn. However, this turn-like motif is located near the N-terminal part in TMP. Secondary structure prediction using Chau-Fasman method suggests that the C-terminal half of the peptide has a tendency to adopt a helical conformation. Together with the highly conserved Trp at position 9, this C-terminal helix may contribute to the stabilization of the dimeric structure. It is interesting to note that most of our tandem repeats are more potent than the C-terminal parallel dimer. Tandem repeats seem to give the molecule a better fit conformation than does the C-C parallel dimerization. The seemingly asymmetric feature of a tandem repeat might have brought it closer to the natural ligand which, as an asymmetric molecule, uses two different sites to bind two identical receptor molecules.

Introduction of a PEG moiety was envisaged to enhance the *in vivo* activity of the modified peptide by providing it a protection against proteolytic degradation and by slowing down its clearance through renal filtration. It was unexpected that pegylation could further increase the *in vitro* bioactivity of a tandem repeated TMP peptide in the cell-based proliferation assay.

## Example 2

### Fc-TMP fusions

TMPs (and EMPs as described in Example 3) were expressed in either monomeric or dimeric form as either N-terminal or C-terminal fusions to the Fc region of human IgG1. In all cases, the expression construct utilized the luxPR promoter promoter in the plasmid expression vector pAMG21.

Fc-TMP. A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a monomer of the TPO-mimetic peptide was constructed using standard PCR technology. Templates for PCR reactions were the pFc-A3 vector and a synthetic TMP gene. The synthetic gene was

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constructed from the 3 overlapping oligonucleotides (SEQ ID NOS: 364, 365, and 366, respectively) shown below:

1842-97      AAA AAA GGA TCC TCG AGA TTA AGC ACG AGC AGC CAG CCA  
CTG ACG CAG AGT CGG ACC  
5  
1842-98      AAA GGT GGA GGT GGT GGT ATC GAA GGT CCG ACT CTG CGT  
1842-99      CAG TGG CTG GCT GCT CGT GCT TAA TCT CGA GGA TCC TTT  
TTT

10

These oligonucleotides were annealed to form the duplex encoding an amino acid sequence (SEQ ID NOS: 367 and 368, respectively) shown below:

15      AAAGGTGGAGGTGGTGGTATCGAAGGTCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCT  
1      -----+-----+-----+-----+-----+ 60  
a      K G G G G I E G P T L R Q W L A A R A -  
20      TAATCTCGAGGATCCTTTTTT  
61      +-----+-----+-----+-----+ 81  
a      ATTAGAGCTCCTAGGAAAAAA  
\*

25 This duplex was amplified in a PCR reaction using 1842-98 and 1842-97 as the sense and antisense primers.

The Fc portion of the molecule was generated in a PCR reaction with pFc-A3 using the primers shown below (SEQ ID NOS: 369 and 370):

30 1216-52      AAC ATA AGT ACC TGT AGG ATC G  
1830-51      TTCGATACCA CCACCTCCAC CTTTACCCGG AGACAGGGAG AGGCCTCTCTGC

The oligonucleotides 1830-51 and 1842-98 contain an overlap of 24  
35 nucleotides, allowing the two genes to be fused together in the correct reading frame by combining the above PCR products in a third reaction using the outside primers, 1216-52 and 1842-97.

The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated  
40 into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described for EMP-Fc herein. Clones were screened for the ability to produce the recombinant protein product and to possess the



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gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #3728.

The nucleotide and amino acid sequences (SEQ ID NOS: 5 and 6) of the fusion protein are shown in Figure 7.

- 5        Fc-TMP-TMP. A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a dimer of the TPO-mimetic peptide was constructed using standard PCR technology. Templates for PCR reactions were the pFc-A3 vector and a synthetic TMP-TMP gene. The synthetic gene was constructed from the 4 overlapping oligonucleotides (SEQ ID NOS: 371 to 374, respectively) shown below:

```

1830-52      AAA GGT GGA GGT GGT GGT ATC GAA GGT CCG
              ACT CTG CGT CAG TGG CTG GCT GCT CGT GCT
15  1830-53      ACC TCC ACC ACC AGC ACG AGC AGC CAG
              CCA CTG ACG CAG AGT CCG ACC
20  1830-54      GGT GGT GGA GGT GGC GGC GGA GGT ATT GAG GGC CCA ACC
              CTT GGC CAA TGG CTT GCA GCA CGC GCA
20  1830-55      AAA AAA AGG ATC CTC GAG ATT ATG CGC GTG CTG CAA GCC
              ATT GGC GAA GGG TTG GGC CCT CAA TAC CTC CGC CGC C

```

- 25        The 4 oligonucleotides were annealed to form the duplex encoding an amino acid sequence (SEQ ID NOS: 375 and 376, respectively) shown below:

```

30  a      1  AAAGGTGGAGGTGGTGGTATCGAAGGTCGACTCTGCGTCAGTGGCTGGCTGCTCGTGCT 60
              -----+-----+-----+-----+-----+
              CCAGGCTGAGACGCAGTCACCGACCGACGACGACGA
              K G G G G G I E G F T L R Q W L A A R A -
35  a      61  GGTGGTGGAGGTGGCGGCGGAGGTATTGAGGGCCCAACCCTTCGCCAATGGCTTGCAGCA 120
              -----+-----+-----+-----+
              CCACCACCTCCACCGCGCCTCCATAACTCCCGGGTTGGGAAGCGGTTACCGAAGTCGT
              G G G G G G G I E G F T L R Q W L A A -
              CGCGCA
40  a      121 -----148
              GCCCGTATTAGAGCTCCTAGGAAAAAAA
              R A *-

```

- 45        This duplex was amplified in a PCR reaction using 1830-52 and 1830-55 as the sense and antisense primers.

The Fc portion of the molecule was generated in a PCR reaction with pFc-A3 using the primers 1216-52 and 1830-51 as described above for

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Fc-TMP. The full length fusion gene was obtained from a third PCR reaction using the outside primers 1216-52 and 1830-55.

The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described in example 1. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #3727.

The nucleotide and amino acid sequences (SEQ ID NOS: 7 and 8) of the fusion protein are shown in Figure 8.

TMP-TMP-Fc. A DNA sequence coding for a tandem repeat of the TPO-mimetic peptide fused in-frame to the Fc region of human IgG1 was constructed using standard PCR technology. Templates for PCR reactions were the EMP-Fc plasmid from strain #3688 (see Example 3) and a synthetic gene encoding the TMP dimer. The synthetic gene for the tandem repeat was constructed from the 7 overlapping oligonucleotides shown below (SEQ ID NOS: 377 to 383, respectively):

20	1885-52	TTT TTT CAT ATG ATC GAA GGT CCG ACT CTG CGT CAG TGG
	1885-53	AGC ACG AGC AGC CAG CCA CTG ACG CAG AGT CGG ACC TTC
		GAT CAT ATG
25	1885-54	CTG GCT GCT CGT GCT GGT GGA GGC GGT GGG GAC AAA ACT
		CAC ACA
	1885-55	CTG GCT GCT CGT GCT GGC GGT GGT GGC GGA GGG GGT GGC
		ATT GAG GGC CCA
30	1885-56	AAG CCA TTG GCG AAG GGT TGG GCC CTC AAT GCC ACC CCC
		TCC GCC ACC ACC GCC
35	1885-57	ACC CTT CGC CAA TGG CTT GCA GCA CGC GCA GGG GGA GGC
		GGT GGG GAC AAA ACT
	1885-58	CCC ACC GCC TCC CCC TGC GCG TGC TGC

These oligonucleotides were annealed to form the duplex shown encoding an amino acid sequence shown below (SEQ ID NOS 384 and 385):

1 TTTTTCATATGATCGAAGGTCCGACTCTGCGTCAGTGGCTGGCGTGCCTGCTGGCGGT 60  
 a GTATACTAGCTTCCAGGCTGAGACGCAGTCACCGACGAGCAGCAGCCGCCA  
 M I E G P T L R Q W L A A R A G G  
 5  
 61 GGTGCGCAGGGGGTGGCATTTAGAGGCCCAACCTTCGCCAATGGCTGGCTGCTCGTCT 120  
 a CCACCGCCTCCCCACCCTAACTCCCGGTTGGGAGCGGTTACCGAACCTCGTGCGCT  
 G G G G G G A I E G P T L R Q W L A A R A -  
 10  
 121 GGTGAGGCGGTGGGACAAACTCTGGCTGCTCGTCTGCTGGAGCGGTGGGGACAAA 180  
 a CCCCCCTCCGCCACCC  
 G G G G G G D K T L A A R A G G G G G D K -  
 15  
 181 ACTCACACA 189  
 a T H T -  
 20

The Fc portion of the molecule was generated in a PCR reaction with DNA from the EMP-Fc fusion strain #3688 (see Example 3) using the primers 1885-54 and 1200-54. The full length fusion gene was obtained from a third PCR reaction using the outside primers 1885-52 and 1200-54.

The nucleotide and amino acid sequences (SEQ ID NOS: 9 and 10)  
35 of the fusion protein are shown in Figure 9.

TMP-Fc. A DNA sequence coding for a monomer of the TPO-mimetic peptide fused in-frame to the Fc region of human IgG1 was obtained fortuitously in the ligation in TMP-TMP-Fc, presumably due to the ability of primer 1885-54 to anneal to 1885-53 as well as to 1885-58. A  
40 single clone having the correct nucleotide sequence for the TMP-Fc construct was selected and designated Amgen strain #3788.

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The nucleotide and amino acid sequences (SEQ ID NOS: 11 and 12) of the fusion protein are shown in Figure 10.

5        Expression in *E. coli*. Cultures of each of the pAMG21-Fc-fusion constructs in *E. coli* GM221 were grown at 37 °C in Luria Broth medium containing 50 mg/ml kanamycin. Induction of gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 20 ng/ml. Cultures were incubated at 37 °C for a further 3 hours. After 3 hours, the bacterial  
10        cultures were examined by microscopy for the presence of inclusion bodies and were then collected by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that the Fc-fusions were most likely produced in the insoluble fraction in *E. coli*. Cell pellets were lysed directly by resuspension in Laemmli sample buffer containing  
15        10% b-mercaptoethanol and were analyzed by SDS-PAGE. In each case, an intense coomassie-stained band of the appropriate molecular weight was observed on an SDS-PAGE gel.

pAMG21. The expression plasmid pAMG21 can be derived from the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be  
20        derived from the Amgen expression vector system described in US Patent No. 4,710,473. The pCFM1656 plasmid can be derived from the described pCFM836 plasmid (Patent No. 4,710,473) by:

- (a) destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end  
25        ligation;
- (b) replacing the DNA sequence between the unique AatII and ClaI restriction sites containing the synthetic P<sub>L</sub> promoter with a similar fragment obtained from pCFM636 (patent No. 4,710,473) containing the PL promoter (see SEQ ID NO: 386 below); and

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(c) substituting the small DNA sequence between the unique ClaI and KpnI restriction sites with the oligonucleotide having the sequence of SEQ ID NO: 388.

SEQ ID NO: 386:

5' AatII  
5' CTAATTCCGCTCTCACCTACCAAACAATGCCCCCTGCAAAAAATAAATTCATAT-  
3' TGCAGATTAAGCGAGAGTGGATGGTTTGTACGGGGGGACGTTTTTTATTTAAGTATA-  
-AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGCGGTGTTGACATAAA-  
10 -TTTTTTGTATGTCATTGTTAGACGCCACTATTTAATAGAGACCGCCACAACGTATTT-  
-TACCACTGGCGGTGATACTGAGCACAT 3'  
-ATGGTGACCGCCACTATGACTCGTGTAGC 5'  
15 ClaI

SEQ ID NO: 387:

5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC 3'  
3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC 5'  
20 ClaI KpnI

The expression plasmid pAMG21 can then be derived from pCFM1656 by making a series of site-directed base changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the BglII site (plasmid bp # 180) immediately 5' to the plasmid replication promoter (25 P<sub>cop</sub>B and proceeding toward the plasmid replication genes, the base pair changes are as shown in Table B below.

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**Table B—Base pair changes resulting in pAMG21**

	<u>pAMG21 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG21</u>
5	# 204	T/A	C/G
	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	--	insert two G/C bp
	# 679	G/C	T/A
10	# 980	T/A	C/G
	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
15	# 1047	C/G	T/A
	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
20	# 2480	A/T	T/A
	# 2499-2502	<u>AGTG</u> TCAC	<u>GTCA</u> CAGT
25	# 2642	<u>TCCGAGC</u> AGGCTCG	7 bp deletion
	# 3435	G/C	A/T
	# 3446	G/C	A/T
30	# 3643	A/T	T/A

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the DNA sequence (SEQ ID NO: 23) shown in Figures 17A and 17B. During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

GM221 (Amgen #2596). The Amgen host strain #2596 is an E.coli K-12 strain derived from Amgen strain #393. It has been modified to contain both the temperature sensitive lambda repressor cI857s7 in the early ebg region and the lacI<sup>q</sup> repressor in the late ebg region (68 minutes). The presence of these two repressor genes allows the use of this host with a variety of expression systems, however both of these repressors are irrelevant to the expression from luxP<sub>g</sub>. The untransformed host has no antibiotic resistances.

The ribosome binding site of the cI857s7 gene has been modified to include an enhanced RBS. It has been inserted into the ebg operon between nucleotide position 1170 and 1411 as numbered in Genbank accession number M64441Cb\_Ba with deletion of the intervening ebg sequence. The sequence of the insert is shown below with lower case letters representing the ebg sequences flanking the insert shown below (SEQ ID NO: 388):

```

ttatttttcgtgCGGCCGACCATTATCACCGCCAGAGGTAACTAGTCAACACGCACGGTCTTAGATATTAT
CCCTTGGCGTGATAGATTGAGCACATCGATTTGATTCAGAAGGAGGGATAATATATGAGCACAAAAAGAAA
CCATTAAACACAAGAGACGCTTGAGGAGCGCACGTCGCTTAAAGCAATTTATGAAAAAAGAAAAATGAACTTG
GCTTATCCAGGAATCTGTCGACAGACAAGATGGGGATGGGGCAGTCAGGCGTTGGTGCTTTATTTAATGGCAT
CAATGCATTAAATGCTTATAACGCCGCGATTGCTTACAAAAAATTCCTCAAGTTAGCGTTGAAGAAATTTAGCCCT
TCAATCGCCAGAGAATCTACGAGATGTATGAAGCGGTTAGTATGCAGCCGTCACCTTAGAAGTGAGTATGAGTA
CCCTGTTTTTCTCAATGTTGAGGACAGGATGTTCTACCTTAAGCTTAGAACCTTTACCAAGGTGATGTCGGAG
AGTATGGGTAGGCACACCAAAAAACCACTGATTTCTGCAATTCGCTTTCAGGTAAAGTAAATTCCTAGACCG
CAGCAACAGGCTCCAAGCCAAGCTTTCCTGACCGAATGTTAATTCGTTGACCCCTGAGCAGGCTGTTGAGCC
AGGTGATTTCTGCATAGCCAGACTTGGGGGTGATGAGTTTACCTTCAAGAACTGATCAGGGATAGCGGTCAG
GTGTTTTTACAACCACTAAACCCACAGTACCCAAATGATCCCATGCAATGAGAGTTGTTCGTTGTTGGGGAAG
TTATCGTATGTCAGTGGCTGAAGAGACGTTTGGCTGATAGACTAGTGGATCCACTAGTgttttcgtccc

```

The construct was delivered to the chromosome using a recombinant phage called MMeBg-cl857s7enhanced RBS #4 into F'tet/393. After recombination and resolution only the chromosomal insert described

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above remains in the cell. It was renamed F'tet/GM101. F'tet/GM101 was then modified by the delivery of a lacI<sup>Q</sup> construct into the ebg operon between nucleotide position 2493 and 2937 as numbered in the Genbank accession number M64441Gb\_Ba with the deletion of the intervening ebg sequence. The sequence of the insert is shown below with the lower case letters representing the ebg sequences flanking the insert (SEQ ID NO: 389) shown below:

```
ggcggaaaccGAGCTCATCGAATGGTGCAAAACCTTTCGGGGTATGGCATGATAGCGCCCGGAAGAGAGTCA
10 ATTACGGGTGGTGAATGTGAAACAGTAACGTTATACGATGTCGCAGAGTATCGCGGTGTCCTTATCAGACG
GTTTCCCGGGTGGTGAACAGGCCAGCCACGTTTCTGCGAAACGCGGGAAAAAGTCGAAGCGGCGATGGCGG
AGCTGGAATTACATTCCCAACCGCGTGGCACAACTGGCGGGCAACAAGTCGCTCCGTGATTGGCGTTGCCAC
15 CTCCAGTCTGGCCCTGCACGCGCGCTCGCAAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCC
AGCGTGGTGGTGTGCGATGGGTAGAACGAAGCGCGCTCGGAAGCCTGTAAAGCGCGCGGTGCACAATCTTCTCGCGC
AACCGCTCAGTGGGCTGATCATTAACTATTCGCTGGATGACCAAGATGCCATTCGCTGTGGAGCTGCTGCAC
15 TAAATGTTCCGGCTTATTTCTTGATGCTCTGACAGACACCCATCAACAGTATTAATTTCTCCCATGAAGAC
GGTAGCGGACATGGCGGTGGAGCATCTGGTTCGCAATTGGGTCACCAACAATCGCGCTGTGATGCGGGCCCATTA
GTTCTGCTCTCGCGCGCTCTGGCTCTGGCTGGCTGGCATAAATATCTCACTCGCAATCAAAATCAGCCGATAGC
20 GGAACGGGAAGCGCATGGAGTGCATGTCGGTTTCAACAACCATGCAAAATGCTGAATGAGGGCATCGTT
CGCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGCGCGCAATGCGCGCATTAACGAGTCCGCGCTGC
GGCTTGGTCCGGAATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCGGTTAAC
25 CACCATCAAAACGATTTTTCGCTGCTGGGGCAACCCAGCTGGAGCCCTTCTGCGAACTCTCTCAAGGGCCAG
CGGTGAAGGGCAATCAGCTGTTGCGCGCTCACTGGTGAAAGAAACACCCCTGGCGCCCAATACGCAAA
CGGCTCTCCCGCGCGGTGGCCGATTCATTAAATGACAGCTGGCACGACAGGTTTCCGAGTGAAGCGGACA
GTAAGGTACCATAGGATCaggcacagga
```

The construct was delivered to the chromosome using a recombinant phage called AGebg-LacIQ#5 into F'tet/GM101. After recombination and resolution only the chromosomal insert described above remains in the cell. It was renamed F'tet/GM221. The F'tet episome was cured from the strain using acridine orange at a concentration of 25 µg/ml in LB. The cured strain was identified as tetracycline sensitive and was stored as GM221.

#### Expression. Cultures of pAMG21-Fc-TMP-TMP in *E. coli* GM221 in

Luria Broth medium containing 50 µg/ml kanamycin were incubated at 37°C prior to induction. Induction of Fc-TMP-TMP gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 20 ng/ml and cultures were incubated at 37°C for a further 3 hours. After 3 hours, the bacterial



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- cultures were examined by microscopy for the presence of inclusion bodies and were then collected by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that the Fc-TMP-TMP was most likely produced in the insoluble fraction in *E. coli*. Cell pellets
- 5 were lysed directly by resuspension in Laemmli sample buffer containing 10%  $\beta$ -mercaptoethanol and were analyzed by SDS-PAGE. An intense Coomassie stained band of approximately 30kDa was observed on an SDS-PAGE gel. The expected gene product would be 269 amino acids in length and have an expected molecular weight of about 29.5 kDa.
- 10 Fermentation was also carried out under standard batch conditions at the 10 L scale, resulting in similar expression levels of the Fc-TMP-TMP to those obtained at bench scale.

- Purification of Fc-TMP-TMP. Cells are broken in water (1/10) by high pressure homogenization (2 passes at 14,000 PSI) and inclusion
- 15 bodies are harvested by centrifugation (4200 RPM in J-6B for 1 hour). Inclusion bodies are solubilized in 6M guanidine, 50mM Tris, 8mM DTT, pH 8.7 for 1 hour at a 1/10 ratio. The solubilized mixture is diluted 20 times into 2M urea, 50 mM tris, 160mM arginine, 3mM cysteine, pH 8.5. The mixture is stirred overnight in the cold and then concentrated about
- 20 10 fold by ultrafiltration. It is then diluted 3 fold with 10mM Tris, 1.5M urea, pH 9. The pH of this mixture is then adjusted to pH 5 with acetic acid. The precipitate is removed by centrifugation and the supernatant is loaded onto a SP-Sepharose Fast Flow column equilibrated in 20mM NaAc, 100 mM NaCl, pH 5(10mg/ml protein load, room temperature).
- 25 The protein is eluted off using a 20 column volume gradient in the same buffer ranging from 100mM NaCl to 500mM NaCl. The pool from the column is diluted 3 fold and loaded onto a SP-Sepharose HP column in 20 mM NaAc, 150 mM NaCl, pH 5(10 mg/ml protein load, room temperature). The protein is eluted off using a 20 column volume gradient

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in the same buffer ranging from 150 mM NaCl to 400 mM NaCl. The peak is pooled and filtered.

Characterization of Fc-TMP activity. The following is a summary of in vivo data in mice with various compounds of this invention.

5 Mice: Normal female BDF1 approximately 10-12 weeks of age.

Bleed schedule: Ten mice per group treated on day 0, two groups started 4 days apart for a total of 20 mice per group. Five mice bled at each time point, mice were bled a minimum of three times a week. Mice were anesthetized with isoflurane and a total volume of 140-160  $\mu$ l of blood was  
10 obtained by puncture of the orbital sinus. Blood was counted on a Technicon H1E blood analyzer running software for murine blood. Parameters measured were white blood cells, red blood cells, hematocrit, hemoglobin, platelets, neutrophils.

Treatments: Mice were either injected subcutaneously for a bolus  
15 treatment or implanted with 7-day micro-osmotic pumps for continuous delivery. Subcutaneous injections were delivered in a volume of 0.2 ml. Osmotic pumps were inserted into a subcutaneous incision made in the skin between the scapulae of anesthetized mice. Compounds were diluted in PBS with 0.1% BSA. All experiments included one control group,  
20 labeled "carrier" that were treated with this diluent only. The concentration of the test articles in the pumps was adjusted so that the calibrated flow rate from the pumps gave the treatment levels indicated in the graphs.

Compounds: A dose titration of the compound was delivered to  
25 mice in 7 day micro-osmotic pumps. Mice were treated with various compounds at a single dose of 100  $\mu$ g/kg in 7 day osmotic pumps. Some of the same compounds were then given to mice as a single bolus injection.

Activity test results: The results of the activity experiments are shown in Figures 11 and 12. In dose response assays using 7-day micro-

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osmotic pumps, the maximum effect was seen with the compound of SEQ ID NO: 18 was at 100  $\mu\text{g}/\text{kg}/\text{day}$ ; the 10  $\mu\text{g}/\text{kg}/\text{day}$  dose was about 50% maximally active and 1  $\mu\text{g}/\text{kg}/\text{day}$  was the lowest dose at which activity could be seen in this assay system. The compound at 10  $\mu\text{g}/\text{kg}/\text{day}$  dose  
5 was about equally active as 100  $\mu\text{g}/\text{kg}/\text{day}$  unpegylated rHu-MGDF in the same experiment.

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### Example 3

### Fc-EMP fusions

**Fc-EMP.** A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a monomer of the EPO-mimetic peptide was constructed using standard PCR technology. Templates for PCR reactions were a vector containing the Fc sequence (pFc-A3, described in International application WO 97/23614, published July 3, 1997) and a synthetic gene encoding EPO monomer. The synthetic gene for the monomer was constructed from the 4 overlapping oligonucleotides (SEQ ID NOS: 390 to 393, respectively) shown below:

1798-2	TAT	GAA	AGG	TGG	AGG	TGG	TGG	AGG	TAC	TTA	CTC	TTG
	CCA	TTT	CGG	ACC	GCT	AGT	TTG	G				
1798-3	CGT	TTT	GCA	ACC	GCA	AGC	CAG	CGG	GCC	GAA	GTG	GCA
	GTA	AGT	ACC	TCC	ACC	ACC	ACC	TCC	ACC	TTT	CAT	AGA
1798-4	GTT	TAT	AAA	CGC	CAG	GST	GGC	GGC	GGC	GGC	GGT	GGT
	ACC	TGC	TCC	TGT	CAT	TTT						
1798-5	CCA	GGT	CAG	CGC	GCC	ACA	ATG	ACA	GGA	ATA	GGT	ACC
	GCG	GCC	GCG	GCC	ACC	ATG						

The 4 oligonucleotides were annealed to form the duplex encoding an amino acid sequence (SEQ ID NOS: 394 and 395, respectively) shown below:

1 TATGAAGGTGGAGGTGGTGGTGGAGGTACTTACTTTGCCACITGGGCCCGGTGACTTG 60  
TACTTTCACCTCCACCACCCATCCATGAATGAGAAGGTGAAGCCGGGACATGAAC  
b M K G G G G G G T T S C H F G P L T W -  
GGTTTGCAAAACCGAGGTGGCGCGCGCGGCGCGGTGTACTATTCTGTGCTTTT  
61 CCAACAGTTTGGGTGCCACCGCCGCCGCCACCTGTATAGGACATGAACCGGCCGACATGGACC 133  
b V . K . P . O . G . G . G . G . G . G . T . T . Y . S . C . H . F . -

This duplex was amplified in a PCR reaction using

1798-18 GCA GAA GAG CCT CTC CCT GTC TCC GGG TAA  
AGG TGG AGG TGG TGG TGG AGG TAC TTA  
CTC T

and

1798-19 CTA ATT GGA TCC ACG AGA TTA ACC ACC  
CTG CGG TTT GCA A

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as the sense and antisense primers (SEQ ID NOS: 396 and 397, respectively).

The Fc portion of the molecule was generated in a PCR reaction with pFc-A3 using the primers

5  
1216-52            AAC ATA AGT ACC TGT AGG ATC G  
1798-17            AGA GTA AGT ACC TCC ACC ACC ACC TCC ACC TTT ACC CGG  
10                    AGA CAG GGA GAG GCT CTT CTG C

15 which are SEQ ID NOS: 398 and 399, respectively. The oligonucleotides 1798-17 and 1798-18 contain an overlap of 61 nucleotides, allowing the two genes to be fused together in the correct reading frame by combining the above PCR products in a third reaction using the outside primers, 1216-52 and 1798-19.

20 The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 (described below), also digested with XbaI and BamHI. Ligated DNA was transformed into competent host cells of E. coli strain 2596 (GM221, described herein). Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #3718.

25 The nucleotide and amino acid sequence of the resulting fusion protein (SEQ ID NOS: 15 and 16) are shown in Figure 13.

EMP-Fc. A DNA sequence coding for a monomer of the EPO-mimetic peptide fused in-frame to the Fc region of human IgG1 was constructed using standard PCR technology. Templates for PCR reactions were the pFC-A3a vector and a synthetic gene encoding EPO monomer.  
30 The synthetic gene for the monomer was constructed from the 4 overlapping oligonucleotides 1798-4 and 1798-5 (above) and 1798-6 and 1798-7 (SEQ ID NOS: 400 and 401, respectively) shown below:

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1798-6 GGC CCG CTG ACC TGG GTA TGT AAG CCA CAA GGG GGT GGG  
GGA GGC GGG GGG TAA TCT CGA G

5 1798-7 GAT CCT CGA GAT TAC CCC CCG CCT CCC CCA CCC CCT TGT  
GGC TTA CAT AC

The 4 oligonucleotides were annealed to form the duplex encoding an amino acid sequence (SEQ ID NOS: 402 and 403, respectively) shown below:

```

1      GTTTGCAACCGCAGGGTGGCGGCGGCGGCGGTACCTATTCCTGTCATTTTGGC
15  A   V C K P Q G G G G G G G G G T Y S C H F G -
      GTCCACCGCCGCGCCGCCACCATGGATAAGGACAGTAAACCG
      CCGCTGACCTGGGTATGTAAGCCACAAGGGGTGGGGGAGGCGGGGGTAATCTCGAG
20  A   P L T W V C K P Q G G G G G G G *
      GGCGACTGGACCATACATTCGGTTCCTCCACCCCTCGGCCCCCATAGAGCTCCTAG

```

This duplex was amplified in a PCR reaction using

25 1798-21 TTA TTT CAT ATG AAA GGT GGT AAC TAT TCC TGT CAT TTT  
and

30 1798-22 TGG ACA TGT GTG AGT TTT GTC CCC CCC GCC TCC CCC ACC  
CCC T

as the sense and antisense primers (SEQ ID NOS: 404 and 405, respectively).

The Fc portion of the molecule was generated in a PCR reaction with pFc-A3 using the primers

35 1798-23 AGG GGG TGG GGG AGG CGG GGG GGA CAA AAC TCA CAC ATG  
TCC A

and

40 1200-54 GTT ATT GCT CAG CGG TGG CA

which are SEQ ID NOS: 406 and 407, respectively. The oligonucleotides 1798-22 and 1798-23 contain an overlap of 43 nucleotides, allowing the two genes to be fused together in the correct reading frame by combining the above PCR products in a third reaction using the outside primers, 1787-21 and 1200-54.

The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated

into the vector pAMG21 and transformed into competent *E. coli* strain 2596 cells as described above. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #3688.

EMP-EMP-Fc. A DNA sequence coding for a dimer of the EPO-mimetic peptide fused in-frame to the Fc region of human IgG1 was constructed using standard PCR technology. Templates for PCR reactions were the EMP-Fc plasmid from strain #3688 above and a synthetic gene encoding the EPO dimer. The synthetic gene for the dimer was constructed from the 8 overlapping oligonucleotides (SEQ ID NOS:408 to 415, respectively) shown below:

40 The 8 oligonucleotides were annealed to form the duplex encoding an amino acid sequence (SEQ ID NOS: 416 and 417, respectively) shown below:

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```

      GGAGGTACTTACTCTTGCCACTTCGGCCCGCTGACTTGGGTTTGCAAAACCGAGGGTGGC
61  -----+-----+-----+-----+-----+-----+-----+
5   a  CCTCCATGAATGAGAACGGTGAAAGCCGGCGCACTGAACCCAAACGTTTGGCGCTCCACCG
      G G T Y S C H F G P L T W V C K P Q G G -

      GCGGCGCGGCGGTGGTACCTATTCTGTCAATTTGGCCCGCTGACCTGGGTATGTAAG
121 -----+-----+-----+-----+-----+-----+-----+
10  a  CCGCCGCCGCCGCCACCATGGATAAGGACAGTAAACCGGGCGACTGGACCCATACATTC
      G G G G G G T Y S C H F G P L T W V C K -

      CCACAAGGGGTGGGGAGGCGGGGGGGACAAAACTCACACATGTCCA
181 -----+-----+-----+-----+-----+-----+
15  a  GGTGTTCCCCACCCCTCCGCCCCCTGTTTGA
      P Q G G G G G G G D K T H T C P -

```

This duplex was amplified in a PCR reaction using 1869-23 and 1871-79 (shown above) as the sense and antisense primers.

The Fc portion of the molecule was generated in a PCR reaction with strain 3688 DNA using the primers 1798-23 and 1200-54 (shown above).

The oligonucleotides 1871-79 and 1798-23 contain an overlap of 31 nucleotides, allowing the two genes to be fused together in the correct reading frame by combining the above PCR products in a third reaction using the outside primers, 1869-23 and 1200-54.

The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described for Fc-EMP. Clones were screened for ability to produce the recombinant protein product and possession of the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #3813.

The nucleotide and amino acid sequences (SEQ ID NOS: 19 and 20, respectively) of the resulting fusion protein are shown in Figure 15. There is a silent mutation at position 145 (A to G, shown in boldface) such that the final construct has a different nucleotide sequence than the oligonucleotide 1871-72 from which it was derived.

Fc-EMP-EMP. A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a dimer of the EPO-mimetic peptide was



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constructed using standard PCR technology. Templates for PCR reactions were the plasmids from strains 3688 and 3813 above.

The Fc portion of the molecule was generated in a PCR reaction with strain 3688 DNA using the primers 1216-52 and 1798-17 (shown above). The EMP dimer portion of the molecule was the product of a second PCR reaction with strain 3813 DNA using the primers 1798-18 (also shown above) and SEQ ID NO: 418, shown below:

1798-20            CTA ATT GGA TCC TCG AGA TTA ACC CCC TTG TGG CTT ACAT

The oligonucleotides 1798-17 and 1798-18 contain an overlap of 61 nucleotides, allowing the two genes to be fused together in the correct reading frame by combining the above PCR products in a third reaction using the outside primers, 1216-52 and 1798-20.

The final PCR gene product (the full length fusion gene) was digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMC21 and transformed into competent E. coli strain 2596 cells as described for Fc-EMP. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #3822.

The nucleotide and amino acid sequences (SEQ ID NOS: \_\_ and \_\_, respectively) of the fusion protein are shown in Figure 16.

Characterization of Fc-EMP activity. Characterization was carried out in vivo as follows.

Mice: Normal female BDF1 approximately 10-12 weeks of age.

Bleed schedule: Ten mice per group treated on day 0, two groups started 4 days apart for a total of 20 mice per group. Five mice bled at each time point, mice were bled a maximum of three times a week. Mice were anesthetized with isoflurane and a total volume of 140-160 ml of blood was obtained by puncture of the orbital sinus. Blood was counted

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on a Technicon H1E blood analyzer running software for murine blood. Parameters measured were WBC, RBC, HCT, HGB, PLT, NEUT, LYMPH.

- Treatments: Mice were either injected subcutaneously for a bolus treatment or implanted with 7 day micro-osmotic pumps for continuous delivery. Subcutaneous injections were delivered in a volume of 0.2 ml. Osmotic pumps were inserted into a subcutaneous incision made in the skin between the scapulae of anesthetized mice. Compounds were diluted in PBS with 0.1% BSA. All experiments included one control group, labeled "carrier" that were treated with this diluent only. The concentration of the test articles in the pumps was adjusted so that the calibrated flow rate from the pumps gave the treatment levels indicated in the graphs.

- Experiments: Various Fc-conjugated EPO mimetic peptides (EMPs) were delivered to mice as a single bolus injection at a dose of 100 µg/kg. Fc-EMPs were delivered to mice in 7-day micro-osmotic pumps. The pumps were not replaced at the end of 7 days. Mice were bled until day 51 when HGB and HCT returned to baseline levels.

#### Example 4

##### TNF-α inhibitors

- Fc-TNF-α inhibitors. A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a monomer of the TNF-α inhibitory peptide was constructed using standard PCR technology. The Fc and 5 glycine linker portion of the molecule was generated in a PCR reaction with DNA from the Fc-EMP fusion strain #3718 (see Example 3) using the sense primer 1216-52 and the antisense primer 2295-89 (SEQ ID NOS: 1112 and 1113, respectively). The nucleotides encoding the TNF-α inhibitory peptide were provided by the PCR primer 2295-89 shown below:

- |         |   |
|---------|---|
| 1216-52 | AAC ATA AGT ACC TGT AGG ATC G                               |
| 2295-89 | CCG CGG ATC CAT TAC GGA CGG TGA CCC AGA GAG GTG TTT TTG TAG |

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TGC GGC AGG AAG TCA CCA CCA CCT CCA CCT TTA CCC

- The oligonucleotide 2295-89 overlaps the glycine linker and Fc portion of the template by 22 nucleotides, with the PCR resulting in the two genes  
5 being fused together in the correct reading frame.

- The PCR gene product (the full length fusion gene) was digested with restriction endonucleases NdeI and BamHI, and then ligated into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described for EMP-Fc herein. Clones were screened for the ability to  
10 produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #4544.

The nucleotide and amino acid sequences (SEQ ID NOS: 1055 and 1056) of the fusion protein are shown in Figures 19A and 19B.

- 15 TNF- $\alpha$  inhibitor-Fc. A DNA sequence coding for a TNF- $\alpha$  inhibitory peptide fused in-frame to the Fc region of human IgG1 was constructed using standard PCR technology. The template for the PCR reaction was a plasmid containing an unrelated peptide fused via a five glycine linker to Fc. The nucleotides encoding the TNF- $\alpha$  inhibitory peptide were  
20 provided by the sense PCR primer 2295-88, with primer 1200-54 serving as the antisense primer (SEQ ID NOS: 1117 and 407, respectively). The primer sequences are shown below:

25 2295-88 GAA TAA CAT ATG GAC TTC CTG CCG CAC TAC AAA AAC ACC TCT CTG GGT  
CAC CGT CCG GGT GGA GGC GGT GGG GAC AAA ACT

30 1200-54 GTT ATT GCT CAG CGG TGG CA

The oligonucleotide 2295-88 overlaps the glycine linker and Fc portion of the template by 24 nucleotides, with the PCR resulting in the two genes being fused together in the correct reading frame.

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The PCR gene product (the full length fusion gene) was digested with restriction endonucleases NdeI and BamHI, and then ligated into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described for EMP-Fc herein. Clones were screened for the ability to

produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #4543.

The nucleotide and amino acid sequences (SEQ ID NOS: 1057 and 1058) of the fusion protein are shown in Figures 20A and 20B.

Expression in E. coli. Cultures of each of the pAMG21-Fc-fusion constructs in E. coli GM221 were grown at 37 °C in Luria Broth medium containing 50 mg/ml kanamycin. Induction of gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 20 ng/ml. Cultures were incubated at 37 °C for a further 3 hours. After 3 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies and were then collected by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that the Fc-fusions were most likely produced in the insoluble fraction in E. coli. Cell pellets were lysed directly by resuspension in Laemmli sample buffer containing 10%  $\beta$ -mercaptoethanol and were analyzed by SDS-PAGE. In each case, an intense coomassie-stained band of the appropriate molecular weight was observed on an SDS-PAGE gel.

Purification of Fc-peptide fusion proteins. Cells are broken in water (1/10) by high pressure homogenization (2 passes at 14,000 PSI) and inclusion bodies are harvested by centrifugation (4200 RPM in J-6B for 1 hour). Inclusion bodies are solubilized in 6M guanidine, 50mM Tris, 8mM DTT, pH 8.7 for 1 hour at a 1/10 ratio. The solubilized mixture is diluted

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20 times into 2M urea, 50 mM tris, 160mM arginine, 3mM cysteine, pH 8.5. The mixture is stirred overnight in the cold and then concentrated about 10 fold by ultrafiltration. It is then diluted 3 fold with 10mM Tris, 1.5M urea, pH 9. The pH of this mixture is then adjusted to pH 5 with acetic acid. The precipitate is removed by centrifugation and the supernatant is loaded onto a SP-Sepharose Fast Flow column equilibrated in 20mM NaAc, 100 mM NaCl, pH 5 (10mg/ml protein load, room temperature). The protein is eluted from the column using a 20 column volume gradient in the same buffer ranging from 100mM NaCl to 500mM NaCl. The pool from the column is diluted 3 fold and loaded onto a SP-Sepharose HP column in 20mM NaAc, 150mM NaCl, pH 5(10mg/ml protein load, room temperature). The protein is eluted using a 20 column volume gradient in the same buffer ranging from 150mM NaCl to 400mM NaCl. The peak is pooled and filtered.

15 Characterization of activity of Fc-TNF- $\alpha$  inhibitor and TNF- $\alpha$  inhibitor-Fc. Binding of these peptide fusion proteins to TNF-  $\alpha$  can be characterized by BIAcore by methods available to one of ordinary skill in the art who is armed with the teachings of the present specification.

#### Example 5

#### 20 IL-1 Antagonists

Fc-IL-1 antagonist. A DNA sequence coding for the Fc region of human IgG1 fused in-frame to a monomer of an IL-1 antagonist peptide was constructed using standard PCR technology. The Fc and 5 glycine linker portion of the molecule was generated in a PCR reaction with DNA from the Fc-EMP fusion strain #3718 (see Example 3) using the sense primer 1216-52 and the antisense primer 2269-70 (SEQ ID NOS: 1112 and 1118, respectively). The nucleotides encoding the IL-1 antagonist peptide were provided by the PCR primer 2269-70 shown below:

30 1216-52 AAC ATA AGT ACC TGT AGG ATC G

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2269-70            CCG CGG ATC CAT TAC AGC GGC AGA GCG TAC GGC TGC CAG TAA CCC  
                     GGG GTC CAT TCG AAA CCA CCA CCT CCA CCT TTA CCC

5

The oligonucleotide 2269-70 overlaps the glycine linker and Fc portion of the template by 22 nucleotides, with the PCR resulting in the two genes being fused together in the correct reading frame.

10            The PCR gene product (the full length fusion gene) was digested with restriction endonucleases NdeI and BamHI, and then ligated into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described for EMP-Fc herein. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected  
15            and designated Amgen strain #4506.

The nucleotide and amino acid sequences (SEQ ID NOS: 1059 and 1060) of the fusion protein are shown in Figures 21A and 21B.

IL-1 antagonist-Fc. A DNA sequence coding for an IL-1 antagonist peptide fused in-frame to the Fc region of human IgG1 was constructed  
20            using standard PCR technology. The template for the PCR reaction was a plasmid containing an unrelated peptide fused via a five glycine linker to Fc. The nucleotides encoding the IL-1 antagonist peptide were provided by the sense PCR primer 2269-69, with primer 1200-54 serving as the antisense primer (SEQ ID NOS: 1119 and 407, respectively). The primer  
25            sequences are shown below:

2269-69            GAA TAA CAT ATG TTC GAA TGG ACC CCG GGT TAC TGG CAG CCG TAC GCT  
                     CTG CCG CTG GGT GGA GGC GGT GGG GAC AAA ACT  
30            1200-54            GTT ATT GCT CAG CGG TGG CA

                 The oligonucleotide 2269-69 overlaps the glycine linker and Fc portion of the template by 24 nucleotides, with the PCR resulting in the two genes  
35            being fused together in the correct reading frame.

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- The PCR gene product (the full length fusion gene) was digested with restriction endonucleases NdeI and BamHI, and then ligated into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described for EMP-Fc herein. Clones were screened for the ability to
- 5 produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #4505.

- The nucleotide and amino acid sequences (SEQ ID NOS: 1061 and 1062) of the fusion protein are shown in Figures 22A and 22B. Expression
- 10 and purification were carried out as in previous examples.

- Characterization of Fc-IL-1 antagonist peptide and IL-1 antagonist peptide-Fc activity. IL-1 Receptor Binding competition between IL-1 $\beta$ , IL-1RA and Fc-conjugated IL-1 peptide sequences was carried out using the IGEN system. Reactions contained 0.4 nM biotin-IL-1R + 15 nM IL-1-TAG
- 15 + 3  $\mu$ M competitor + 20  $\mu$ g/ml streptavidin-conjugate beads, where competitors were IL-1RA, Fc-IL-1 antagonist, IL-1 antagonist-Fc). Competition was assayed over a range of competitor concentrations from 3  $\mu$ M to 1.5 pM. The results are shown in Table C below:

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**Table C—Results from IL-1 Receptor Binding Competition Assay**

		<i>IL-1pep-Fc</i>	<i>Fc-IL-1pep</i>	<i>IL-1ra</i>
5	KI EC50	281.5 530.0	59.58 112.2	1.405 2.645
	<b>95% Confidence Intervals</b>			
10	EC50	280.2 to 1002	54.75 to 229.8	1.149 to 6.086
	KI	148.9 to 532.5	29.08 to 122.1	0.6106 to 3.233
15	<b>Goodness of Fit</b>			
	R <sup>2</sup>	0.9790	0.9687	0.9602



**Example 6****VEGF-Antagonists**

**Fc-VEGF Antagonist.** A DNA sequence coding for the Fc region of

- 5 human IgG1 fused in-frame to a monomer of the VEGF mimetic peptide was constructed using standard PCR technology. The templates for the PCR reaction were the pFc-A3 plasmid and a synthetic VEGF mimetic peptide gene. The synthetic gene was assembled by annealing the following two oligonucleotides primer (SEQ ID NOS: 1120 and 1121, respectively):

- 10 2293-11 GTT GAA CCG AAC TGT GAC ATC CAT GTT ATG TGG GAA TGG GAA  
TGT TTT GAA CGT CTG
- 15 2293-12 CAG ACG TTC AAA ACA TTC CCA TTC CCA CAT AAC ATG GAT GTC  
ACA GTT CGG TTC AAC

The two oligonucleotides anneal to form the following duplex encoding an amino acid sequence shown below (SEQ ID NOS 1122):

- 20
- ```

      GTTGAACCGAACTGTGACATCCATGTTATGTGGGAATGGGAATGTTTGAACGTCGTG
1  -----+-----+-----+-----+-----+----- 57
      CAACTTGGCTTGACACTGTAGGTACAATACACCCTTACCCTTACAAAACCTGCAGAC

```
- 25 a V E P N C D I H V M W E W E C F E R L  
-

This duplex was amplified in a PCR reaction using 2293-05 and 2293-06 as the sense and antisense primers (SEQ ID NOS. 1125 and 1126).

- 30 The Fc portion of the molecule was generated in a PCR reaction with the pFc-A3 plasmid using the primers 2293-03 and 2293-04 as the sense and antisense primers (SEQ ID NOS. 1123 and 1124, respectively). The full length fusion gene was obtained from a third PCR reaction using the outside primers 2293-03 and 2293-06. These primers are shown below:

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2293-03     ATT TGA TTC TAG AAG GAG GAA TAA CAT ATG GAC AAA ACT CAC  
ACA TGT

5     2293-04     GTC ACA GTT CGG TTC AAC ACC ACC ACC ACC ACC TTT ACC CGG  
AGA CAG GGA

2293-05     TCC CTG TCT CCG GGT AAA GGT GGT GGT GGT GTT GAA CCG  
AAC TGT GAC ATC

10     2293-06     CCG CGG ATC CTC GAG TTA CAG ACG TTC AAA ACA TTC CCA

The PCR gene product (the full length fusion gene) was digested with restriction endonucleases NdeI and BamHI, and then ligated into the vector pAMG21 and transformed into competent E. coli strain 2596 cells as described for EMP-Fc herein. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. A single such clone was selected and designated Amgen strain #4523.

20     The nucleotide and amino acid sequences (SEQ ID NOS: 1063 and 1064) of the fusion protein are shown in Figures 23A and 23B.

VEGF antagonist -Fc. A DNA sequence coding for a VEGF mimetic peptide fused in-frame to the Fc region of human IgG1 was constructed using standard PCR technology. The templates for the PCR reaction were the pFc-A3 plasmid and the synthetic VEGF mimetic peptide gene described above. The synthetic duplex was amplified in a PCR reaction using 2293-07 and 2293-08 as the sense and antisense primers (SEQ ID NOS. 1127 and 1128, respectively).

30     The Fc portion of the molecule was generated in a PCR reaction with the pFc-A3 plasmid using the primers 2293-09 and 2293-10 as the sense and antisense primers (SEQ ID NOS. 1129 and 1130, respectively).

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The full length fusion gene was obtained from a third PCR reaction using the outside primers 2293-07 and 2293-10. These primers are shown below:

5      2293-07      ATT TGA TTC TAG AAG GAG GAA TAA CAT ATG GTT GAA CCG AAC  
                 TGT GAC

                 2293-08      ACA TGT GTG AGT TTT GTC ACC ACC ACC ACC ACC CAG ACG TTC  
                 AAA ACA TTC

10      2293-09      GAA TGT TTT GAA CGT CTG GGT GGT GGT GGT GGT GAC AAA ACT  
                 CAC ACA TGT

                 2293-10      CCG CGG ATC CTC GAG TTA TTT ACC CGG AGA CAG GGA GAG

                 The PCR gene product (the full length fusion gene) was digested  
15      with restriction endonucleases NdeI and BamHI, and then ligated into the  
vector pAMG21 and transformed into competent E. coli strain 2596 cells as  
described for EMP-Fc herein. Clones were screened for the ability to  
produce the recombinant protein product and to possess the gene fusion  
having the correct nucleotide sequence. A single such clone was selected  
20      and designated Amgen strain #4524.

                 The nucleotide and amino acid sequences (SEQ ID NOS: 1065 and  
1066) of the fusion protein are shown in Figures 24A and 24B. Expression  
and purification were carried out as in previous examples.

25

### **Example 7**

#### **MMP Inhibitors**

Fc-MMP inhibitor. A DNA sequence coding for the Fc region of  
human IgG1 fused in-frame to a monomer of an MMP inhibitory peptide  
was constructed using standard PCR technology. The Fc and 5 glycine  
30      linker portion of the molecule was generated in a PCR reaction with DNA  
from the Fc-TNF- $\alpha$  inhibitor fusion strain #4544 (see Example 4) using the  
sense primer 1216-52 and the antisense primer 2308-67 (SEQ ID NOS: 1112

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and 1131, respectively). The nucleotides encoding the MMP inhibitor peptide were provided by the PCR primer 2308-67 shown below:

5      1216-52      AAC ATA AGT ACC TGT AGG ATC G  
2308-67      CCG CGG ATC CAT TAG CAC AGG GTG AAA CCC CAG TGG GTG GTG  
                 CAA CCA CCA CCT CCA CCT TTA CCC

The oligonucleotide 2308-67 overlaps the glycine linker and Fc portion of  
10 the template by 22 nucleotides, with the PCR resulting in the two genes  
being fused together in the correct reading frame.

The PCR gene product (the full length fusion gene) was digested  
with restriction endonucleases NdeI and BamHI, and then ligated into the  
vector pAMG21 and transformed into competent E. coli strain 2596 cells as  
15 described for EMP-Fc herein. Clones were screened for the ability to  
produce the recombinant protein product and to possess the gene fusion  
having the correct nucleotide sequence. A single such clone was selected  
and designated Amgen strain #4597.

The nucleotide and amino acid sequences (SEQ ID NOS: 1067 and  
20 1068) of the fusion protein are shown in Figures 25A and 25B. Expression  
and purification were carried out as in previous examples.

MMP Inhibitor-Fc. A DNA sequence coding for an MMP inhibitory  
peptide fused in-frame to the Fc region of human IgG1 was constructed  
using standard PCR technology. The Fc and 5 glycine linker portion of the  
25 molecule was generated in a PCR reaction with DNA from the Fc-TNF- $\alpha$   
inhibitor fusion strain #4543 (see Example 4). The nucleotides encoding  
the MMP inhibitory peptide were provided by the sense PCR primer 2308-  
66, with primer 1200-54 serving as the antisense primer (SEQ ID NOS:  
1132 and 407, respectively). The primer sequences are shown below:

30  
2308-66      GAA TAA CAT ATG TGC ACC ACC CAC TGG GGT TTC ACC CTG TGC  
                 GGT GGA GGC GGT GGG GAC AAA  
35 1200-54      GTT ATT GCT CAG CGG TGG CA



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|    |          |                                                               |
|----|----------|---------------------------------------------------------------|
|    | BSA      | Bovine serum albumin                                          |
|    | Bzl      | Benzyl                                                        |
|    | Cap      | Caproic acid                                                  |
|    | CTL      | Cytotoxic T lymphocytes                                       |
| 5  | CTLA4    | Cytotoxic T lymphocyte antigen 4                              |
|    | DARC     | Duffy blood group antigen receptor                            |
|    | DCC      | Dicyclohexylcarbodiimide                                      |
|    | Dde      | 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)ethyl               |
|    | EMP      | Erythropoietin-mimetic peptide                                |
| 10 | ESI-MS   | Electron spray ionization mass spectrometry                   |
|    | EPO      | Erythropoietin                                                |
|    | Fmoc     | fluorenylmethoxycarbonyl                                      |
|    | G-CSF    | Granulocyte colony stimulating factor                         |
|    | GH       | Growth hormone                                                |
| 15 | HCT      | hematocrit                                                    |
|    | HGB      | hemoglobin                                                    |
|    | hGH      | Human growth hormone                                          |
|    | HOBt     | 1-Hydroxybenzotriazole                                        |
|    | HPLC     | high performance liquid chromatography                        |
| 20 | IL       | interleukin                                                   |
|    | IL-R     | interleukin receptor                                          |
|    | IL-1R    | interleukin-1 receptor                                        |
|    | IL-1ra   | interleukin-1 receptor antagonist                             |
|    | Lau      | Lauric acid                                                   |
| 25 | LPS      | lipopolysaccharide                                            |
|    | LYMPH    | lymphocytes                                                   |
|    | MALDI-MS | Matrix-assisted laser desorption ionization mass spectrometry |
|    | Me       | methyl                                                        |

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|    |       |                                                   |
|----|-------|---------------------------------------------------|
|    | MeO   | methoxy                                           |
|    | MHC   | major histocompatibility complex                  |
|    | MMP   | matrix metalloproteinase                          |
|    | MMPI  | matrix metalloproteinase inhibitor                |
| 5  | 1-Nap | 1-naphthylalanine                                 |
|    | NEUT  | neutrophils                                       |
|    | NGF   | nerve growth factor                               |
|    | Nle   | norleucine                                        |
|    | NMP   | N-methyl-2-pyrrolidinone                          |
| 10 | PAGE  | polyacrylamide gel electrophoresis                |
|    | PBS   | Phosphate-buffered saline                         |
|    | Pbf   | 2,2,4,6,7-pendamethyldihydrobenzofuran-5-sulfonyl |
|    | PCR   | polymerase chain reaction                         |
|    | Pec   | pipecolic acid                                    |
| 15 | PEG   | Poly(ethylene glycol)                             |
|    | pGlu  | pyroglutamic acid                                 |
|    | Pic   | picolinic acid                                    |
|    | PLT   | platelets                                         |
|    | pY    | phosphotyrosine                                   |
| 20 | RBC   | red blood cells                                   |
|    | RBS   | ribosome binding site                             |
|    | RT    | room temperature (25 °C)                          |
|    | Sar   | sarcosine                                         |
|    | SDS   | sodium dodecyl sulfate                            |
| 25 | STK   | serine-threonine kinases                          |
|    | t-Boc | tert-Butoxycarbonyl                               |
|    | tBu   | tert-Butyl                                        |
|    | TGF   | tissue growth factor                              |
|    | THF   | thymic humoral factor                             |

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|    |       |                                         |
|----|-------|-----------------------------------------|
|    | TK    | tyrosine kinase                         |
|    | TMP   | Thrombopoietin-mimetic peptide          |
|    | TNF   | Tissue necrosis factor                  |
|    | TPO   | Thrombopoietin                          |
| 5  | TRAIL | TNF-related apoptosis-inducing ligand   |
|    | Trt   | trityl                                  |
|    | UK    | urokinase                               |
|    | UKR   | urokinase receptor                      |
|    | VEGF  | vascular endothelial cell growth factor |
| 10 | VIP   | vasoactive intestinal peptide           |
|    | WBC   | white blood cells                       |